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14. ABSTRACT
Surgery of the spine to fuse the vertebral bones is one of the most commonly performed operations with an estimated 400,000 Americans to undergo this surgery in the year 2003. It is useful for the treatment of scoliosis, instability and painful degenerative conditions of the spine, but as currently performed is highly invasive and has a low success rate. Often bone must be surgically removed from the pelvis, to implant in the spine for proper healing, which requires an additional surgery. This additional surgery often results in significant pain, and long term healing. We propose to develop a system in which cells carrying an adenovirus making a bone forming protein would be injected through the skin into the location near the spine which requires fusion. The cells used for this experiment could be those isolated from the patients own blood, and would not require any invasive surgical procedures. The first steps to creating such a procedure is to test this type of system in animals and determine the optimal conditions necessary to achieve spine fusion. Validation of our hypothesis will provide a safe and efficacious gene therapy system for the production of bone by providing a cellular delivery system for the bone forming protein and localization of the cells to the spine, circumventing the need for direct administration of the viruses that could have potential systemic side effects.

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Introduction: Surgery of the spine to fuse the vertebral bones is one of the most commonly performed operations with some 400,000 Americans undergoing this type of surgery annually in the United States. The estimated cost associated with such procedures exceeding $60 billion annually demonstrating this to be a significant problem. In the most common form, posterolateral fusion, the paraspinous musculature is stripped and the bone decorticated, resulting in significant pain, reduced stability afforded by these muscles, and disruption of the blood supply to both bone and muscle. Further, success rates for fusion range from 50-70% depending on how many levels are fused and the number and types of attendant complications. We recently demonstrated that transduced cells expressing high levels of bone morphogenetic protein 2 (BMP2) in skeletal muscle could rapidly recruit and expand endogenous cell populations to initiate all stages of endochondral bone formation, with mineralized bone forming within one week of implantation. The central hypothesis of this application is that posterolateral spine fusion can be successfully achieved with only minimally invasive percutaneous techniques and without a scaffold, by collecting cells from patient's, transducing them to express BMP2, encapsulating the cells with hydrogel material, and then delivering them to the fusion site. If added structural stability is required, the injectable hydrogel will be crosslinked *in vivo* with a small fiber-optic light source. Successful completion of this project would advance the current state of gene therapy in this field by eliminating the search for an optimal osteoprogenitor cell and scaffolding.

Body: The central hypothesis of this application is that posterolateral spine fusion can be successfully achieved with only minimally invasive percutaneous techniques and without a scaffold, by collecting cells from patient's peripheral blood, transducing them to express an osteoinductive factor (bone morphogenetic protein 2 or BMP2), encapsulating the cells with hydrogel material, and then delivering them to the fusion site. We have developed three specific tasks to accomplish our goals.

Task 1: To produce high levels of BMP2 from human peripheral blood cells transduced with the Ad5F35BMP2 chimeric adenovirus. (Months 7-14). These tasks were reviewed in our last update. We have included the previous data reported last year, but also provided additional data that has led to a recent publication in *Human Gene Therapy* (Fouletier-Dilling *et al*, 2005). Since our previous studies (first progress report) demonstrated a significant reduction (≥50%) in the amount of BMP2 secreted from human peripheral blood mononuclear cells transduced with adenovirus vectors, as compared to similar numbers of other fibroblast cell lines transduced in parallel, we focused on improving the transduction efficiency. In these studies using a variety of cells rather than human peripheral blood we developed a novel method for improving transduction efficiency with our chimeric virus.

a. ***Determine the viral transduction efficiency of human peripheral blood with Ad5F35 chimeric adenovirus, and compare this to the efficiency of transduction in purified human peripheral blood mononuclear cells. (Months 1-4).*** Briefly, our results indicated that a small amount of BMP2 was detected in the media in both concentrations of purified mononuclear cells, but this was greatly reduced to that which is found with other fibroblasts (Figure 1). However, in the whole blood samples with the same number of white cells, BMP2 activity was equal to the control suggesting that either the vector was not able to efficiently transduce the cells, or the BMP2 was being rapidly degraded. **Results indicate that we must purify mononuclear cells if we continue to use human peripheral blood.**

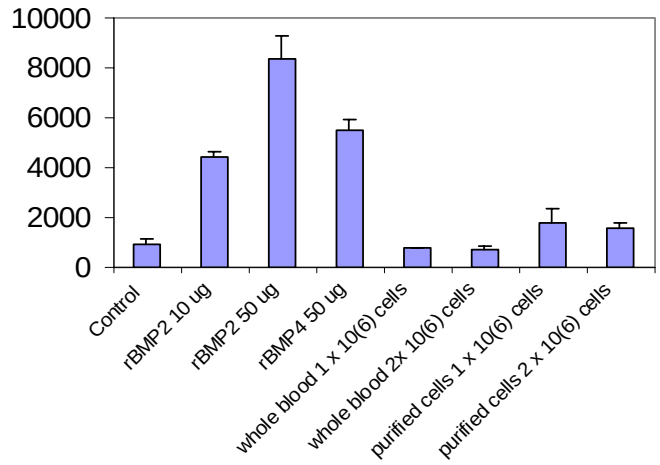


Figure 1: Alkaline phosphatase induction in W20-17 cells after exposure to BMP2. BMP2 activity in was assessed in culture supernatant taken from either whole peripheral or purified mononuclear cells from blood 48 hours after transduction with Ad5F35BMP2 (2500 vp/cell).

b. ***BMP2 levels will be determined by Western blot analysis of human peripheral blood cells transduced with the chimeric adenovirus. (Months 5-8).***

From our results described in the above section, we chose to pursue improving the transduction in purified mononuclear cells. We therefore isolated peripheral blood, purified the mononuclear cells as described above, and plated them directly into a 24 well dish in varying concentrations (10^6,10^7,10^8) in DMEM supplemented with 2% FBS, with antibiotic-antimycotic. Since we previously determined that the critical factor for inducing bone formation is the level of BMP2 secretion (Olmsted-Davis *et al* 2002, Gugala *et al*, 2003) expression by escalating both the cell number and adenovirus dose. Therefore the cells were transduced with Ad5F35BMP2 with varying MOI of (2500 vp/cell, 5000 vp/cell, and 10,000 vp/cell) and placed in a humid chamber at 37°C and 5% CO_2 for 72 hours prior to collection of the culture supernatant. Cells were also transduced with Ad5F35GFP (10,000 vp/cell) as a control. We have chosen to assay the BMP2 by an ELISA (R&D systems, Inc., Minneapolis, MS) rather than western blot in that it is a more rapid, quantitative method for large numbers of samples. Briefly, the culture supernatant was added to the ELISA assay according to manufacturer's specifications. rBMP2, of known concentrations, was used to generate a standard curve which was then used to quantify the amount of BMP2 in culture supernatant. Figure 2, shows the results of the BMP2 quantification.

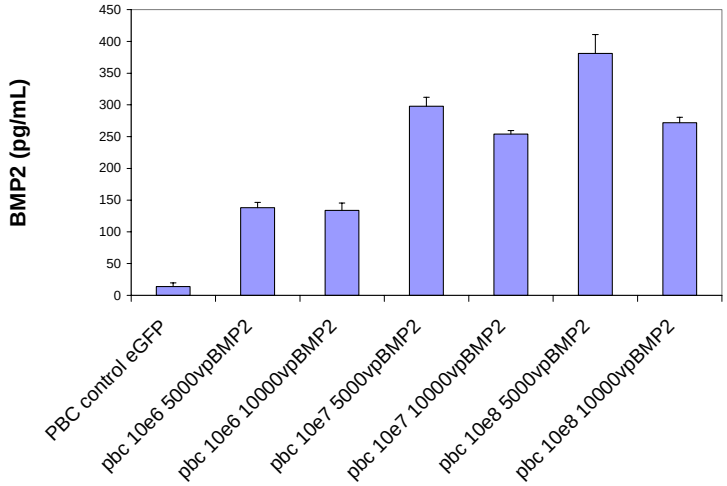


Figure 2: Analysis of BMP2 protein in conditioned media from purified mononuclear cells in peripheral blood. Mononuclear cells were purified on a Ficoll-Paque™ PLUS (Amersham Pharmacia Biotech, Piscatawy, NJ) and transduced with Ad5F35BMP2 virus (5000 vp/cell and 10,000 vp/) or Ad5F35GFP (10,000 vp/cell). Cell number was also varied from 10^6, 10^7, 10^8 cells/ml as indicated and culture supernatant was collected 72 hours after initial transduction. Cell viability in these experiments was determined to be greater than 90%.

As can bee seen in figure 2, escalation of virus dose did not appear to enhance the BMP2 expression, suggesting that the cellular internalization mechanism may be saturated, and hence no more virus can be taken up by the cells. As expected we did see an elevation of BMP2 in culture supernatant with increasing number of transduced cells. However, this level of expression is still significantly lower than what we achieve with the fibroblasts; therefore we may need to consider some alternatives beyond escalation of cell number. Please see the conclusions **section d, *Task 1*** for alternative strategies.

In an attempt to improve the transduction efficiency of the Ad5F35BMP2 we chose to compare the normal adenovirus infection methods to that obtained when the lipid-polyamine known as GeneJammer® Transfection Reagent (Stratagene, La Jolla, CA) was included in the infection. Although this is currently

marketed for DNA rather than virus transfer, we have determined that this reagent greatly enhances the transduction of cells with adenovirus (Fouletier-Dilling *et al*, 2006, see appendix).

- ***Enhanced viral transduction in the presence of GeneJammer®***

Adenovirus transduction of coxackie-adenovirus receptor (CAR)-negative cell lines is extremely inefficient requiring large amounts of virus, and resulting in low level expression of the desired transgene. To enhance virus uptake into the cells, we tested the ability of the commercially available polyamine complex, GeneJammer® to enhance virus transduction. Accordingly, CAR-negative hBM-MSCs (10^6 cells) were transduced with Ad5eGFP virus at three different concentrations (2500 vp/cell, 5000 vp/cell, and 10000 vp/cell) in the presence or absence of GeneJammer® (Fig. 3A). An adenovirus type 5 lacking any transgene (Ad-empty) was used as a negative control. As seen in Fig. 1A, the presence of the polyamine, GeneJammer® increases the number of the hBM-MSCs transduced cells for all concentrations of virus. The lowest MOI of 2500 vp/cell resulted in the most dramatic increase in number of transduced cells, with 15% in the absence of GeneJammer® and 95% transduced in the presence of the polyamine. Further we observed an increase in the number of transduced cells with a corresponding increase in virus MOI in the absence of GeneJammer®, however, in the presence GeneJammer®, the maximum number of transduced cells (95-100%) was found for all virus concentrations.

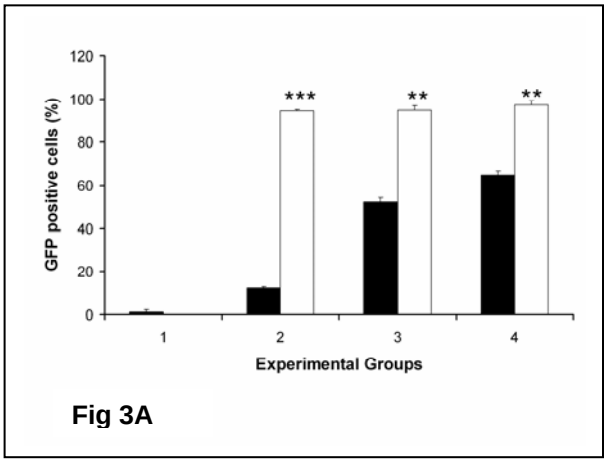
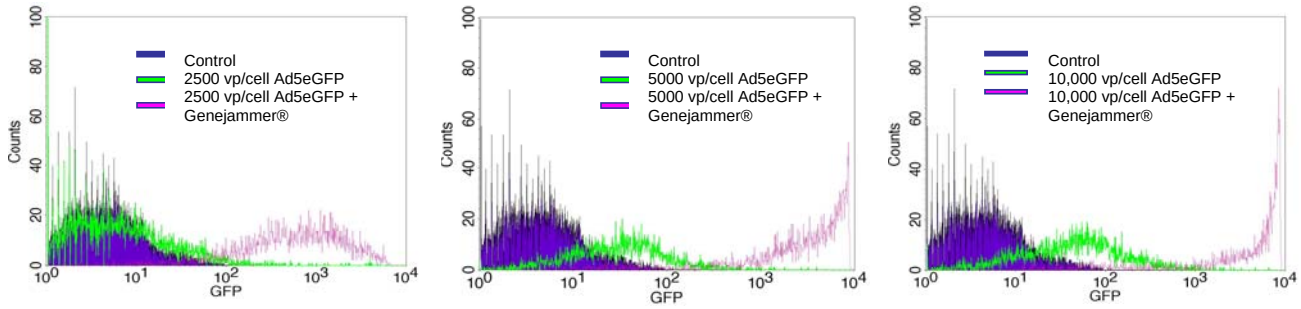


Fig 3A

Since multiple virus particles can enter the same cell, we also analyzed the samples transduced in the presence of GeneJammer®, in which we obtained 95-100% cell transduction, to determine if the intensity of GFP expression increased with virus concentration. As can be seen in Figure 3B, the intensity of GFP expression from the cells transduced with GeneJammer®, increased with virus dose. Since 100% of the cells were transduced at all virus doses in this population, the increase in GFP intensity presumably represents an increase in the number of virus particles each cell is taking up. The data suggests that GeneJammer® not only enhances the number of cells taking up the virus (Fig. 3A) but also the total amount of virus entering any given cell (Fig. 3B).

Figure 3B



Cell Number

Figure 3: (A) Flow cytometric quantification of GFP expression after transduction of hBM-MSCs with (1) Ad5-empty, (2) Ad5eGFP 2,500vp/cell, (3) Ad5eGFP 5,000vp/cell, or (4) Ad5eGFP 10,000vp/cell in the absence (solid columns) or presence (open columns) of GeneJammer®. The percentage of GFP-positive cells is depicted as the average eGFP fluorescence, where *n*=3. Columns and error bars represent means ± standard deviation for *n*=3. *** represent *p*<0.001 and ** represent *p*<0.01 (Student *t* test). **(B)** GFP fluorescence intensity shifts in the flow cytometry profiles of eGFP expression in the hBM-MSCs transduced with either 2,500 vp/cell, 5,000 vp/cell, or 10,000 vp/cell Ad5GFP in the presence of GeneJammer® shown in **(A)**. In all samples 100% of the cells were found to express eGFP.

The compound GeneJammer® allows adenovirus to enter cells lacking the receptor for fiber

Two potential models exist as to the mechanism by which the polyamine enhances virus uptake. First, GeneJammer® may aid in virus binding to its receptor, therefore potentially acting as a co-receptor for the virus internalization. Alternatively, this compound may bind to the virus and promote a novel entry route into the cell. To determine which of these is most likely, we compared the transduction efficiency of various cell types known to have differential expression levels of CAR and αV integrin (Ad5) or CD46 (Ad5F35), in the presence or absence of GeneJammer®. The three cells lines chosen have the following receptor characteristics: A549 cells express high levels of CAR, αV integrin, and CD46; hBM-MSCs lack CAR but express αV integrin, and express moderate levels of CD46, and CHO cells express little to no adenovirus receptors (Table 1). As expected, Ad5eGFP (2500 vp/cell) transduced 100% of the receptor positive A549 cells, while less than 15% of the receptor negative CHO cells were transduced (Fig. 4A and C).

Table 1: Adenovirus receptors expression on selected cell lines

Cell Line	Cell Surface Receptor		
	CAR	**Integrin V**	**CD46**
A549	93.28% ± 1.28	99.26% ± 0.53	99.27% ± 0.79
hBM-MSCs	*0.34% ± 0.50	*87% ± 4.70	31.78% ± 3.54
CHO	1.77% ± 0.27	2.04% ± 0.16	0.19% ± 0.05

Numbers represent mean percentage of cells expressing each receptor SC (n=3)
*Olmsted-Davis *et al* 2002.

The hBM-MSCs which are αV integrin positive-CAR negative yielded approximately 10% of the cells expressing GFP at this same MOI (Fig. 4B). Parallel transductions done in the presence of GeneJammer® showed significant enhancement in transduction in both the cells lacking CAR but expressing αV integrin, as well as the receptor negative cells (Fig. 4B and C). The results (Fig. 4C) show approximately 95% of the transduced CHO cells were expressing GFP when the adenovirus was delivered in the presence of the polyamine, suggesting that the molecular mechanism is not adenovirus receptor associated but rather,

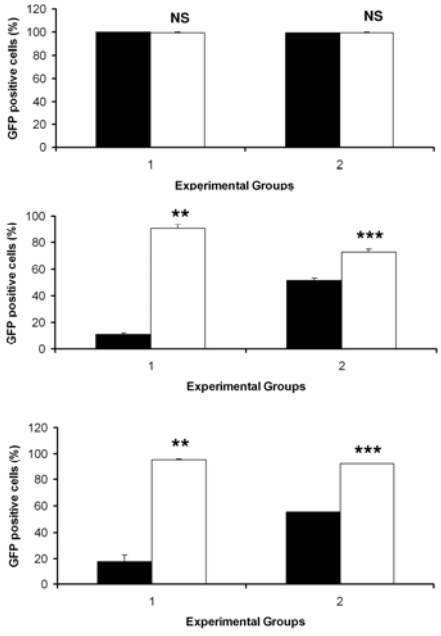


Figure 4

Figure 4: Flow cytometry analysis of GFP expression of A549 cells **(A)**, hBM-MSCs (B) and CHO cells **(C)** transduced with Ad5eGFP 2,500 vp/cell (bar 1), Ad5F35eGFP 2,500 vp/cell (bar 2). In the absence ■ or presence □ of GeneJammer®. The percentage of GFP positive cells was depicted as the average GFP fluorescence where n=3. Errors bars represent means ± standard deviation for n=3. *** represent *p*<0.001 and ** represent *p*<0.01; Student *t* test.

a novel pathway for virus entry into the cell. We did not see any significant changes between the groups in the A549 cells (Fig. 4A) due to the fact that the cells in the absence of GeneJammer® have been maximally transduced. However, the data suggests that these two systems for virus entry do not appear to inhibit one another (Fig. 4A).

Next we chose to determine if the virus entry into the cells via the polyamine was specific to the adenovirus type 5 capsid, so similar experiments were conducted using the altered fiber virus Ad5F35eGFP. The results were similar to those obtained with the Ad5 vector, suggesting similar mechanism that is not dependant on adenovirus type 5 fiber for entry into the cell (Figure 4).

Since the GeneJammer® allowed for adenovirus entry in the absence of receptor, we tested whether it could improve the transduction efficiency of human peripheral blood mononuclear cells. Briefly, the cells were isolated and purified as described above, a 24 well dish was plated at a cell density of 1 x 10^6 cells per well. Cells were then either transduced with Ad5F35GFP, or no additions, in DMEM + 2% FBS and antibiotic-antimycotic for 24 hours, prior to increasing the serum, or in DMEM + 10% FBS and a antibiotic-antimycotic and the 1.2% polyamine GeneJammer® Transfection Reagent (Stratagene, La Jolla, CA) according to manufacturers specifications for approximately 4 hours, prior to addition of more media to dilute the polyamine. The percentage of Ad5F35eGFP positively transduced cells was determined using flow cytometry. Briefly, cells were washed and resuspended in phosphate buffered saline (PBS). Dead cells and debris were excluded from analysis by using propidium iodide (PI). Samples were run on a FACSCalibur cytometer (Becton Dickinson, San Jose, CA). Percentage of cells transduced to express GFP was determined as relative fluorescence intensity (RFI) of the total viable cell population. Cell viability was determined by addition of propidium Iodide (50 µ/mL) to the cells, and detection using the FACScan Analyzer. Transduction of the purified blood mononuclear cells was enhanced as can be seen in figure 5.

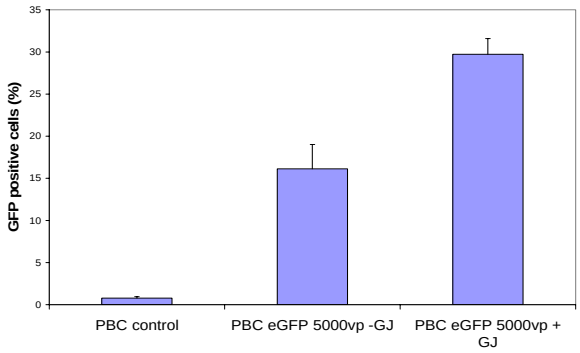


Figure 5: Flow cytommetray analysis of GFP expression in purified mononuclear cells after transduction with Ad5F35GFP in the presence and absence of GeneJammer® Transfection Reagent (Stratagene, La Jolla, CA). Control cells represent those which were not transduced with virus.

Next we looked at BMP2 expression from the peripheral blood mononuclear cells 72 hours after transduction with Ad5F35BMP2 in the presence or absence of GeneJammer®. Media was collected and the level of BMP2 determined by an ELISA assay described above to detect BMP2,

As can be seen in figure 6A, when the polyamine was included during the transduction, there was a significant enhancement (approximately 40%) in BMP2 expression from the same number of cells, hence allowing us to increase the amount of BMP2 being produced per individual cell.

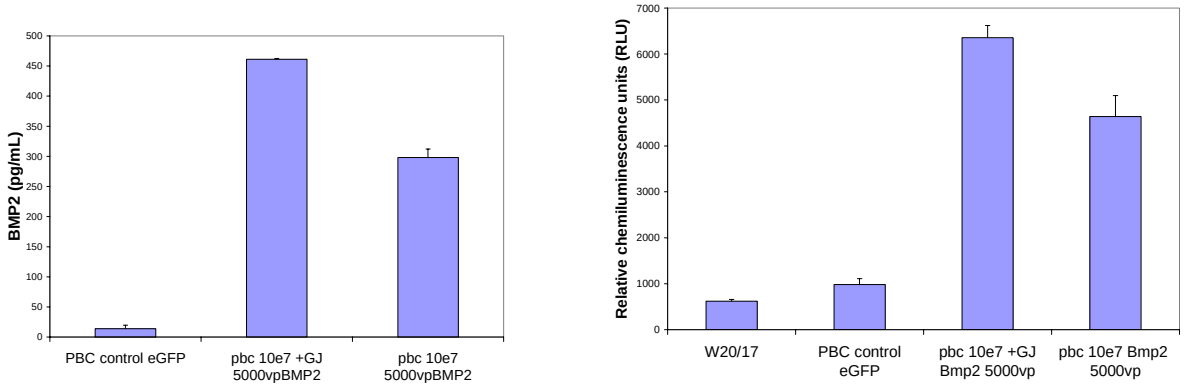


Figure 6: Comparison of standard adenovirus transduction methods versus transduction using GeneJammer® Transfection Reagent (Stratagene, La Jolla, CA). (A) Transduction efficiency of purified mononuclear cells using Ad5F35BMP2 in the presence and absence of the polyamine GeneJammer®. Approximately 10^7 cells were transduced with Ad5F35BMP2 (5000 vp/cell) and 72hrs later, BMP2 was measured in the culture supernatant using an ELISA assay (R&D Systems, Inc. Minneapolis, MN). **(B)** Analysis of BMP2 activity in the media as detected by the level of alkaline phosphatase induction in W20-17 cells after exposure to the culture supernatants collected from cells transduced with either Ad5F35BMP2 or Ad5F35eGFP

To determine the level of BMP2 activity within the culture supernatant after transduction of the purified mononuclear cells with Ad5F35BMP2, we used the murine bone marrow cells known as W20-17. These cells have previously been shown to respond to active BMP2 by elevating the level of alkaline phosphatase activity (Thies *et al.*, 1992). Briefly, culture supernatant from the samples described above, was added to the W20-17 cells, and alkaline phosphatase activity was assayed using a chemiluminescent detection system (Olmsted *et al.*, 2001), (Blum *et al*, 2001). Cellular alkaline phosphatase was extracted by three freeze-thaw cycles in 100 μl/cm^2 of 25mM Tris-HCl, pH 8.0 and 0.5% Triton X-100 and the activity was then measured by addition of CSPD® Ready-to-use with Sapphire II enhancer (Tropix, Bedford, MA) to the samples. The light output from each sample was integrated for 10 seconds after a 2 second delay using a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA). Alkaline phosphatase levels were recorded in relative luminescence units (RLU) and normalized to protein content with the BCA assay using bovine serum albumin to derive a standard curve. Data are presented as percent induction above unstimulated basal control cells.

However, the purified peripheral blood mononuclear cells, still produced significant less BMP2 per 10^7 cell 450 pg/ml as compared to 15,000 pg/ml observed when other fibroblast were transduced in parallel with Ad5F35BMP2 (2500 vp/cell) in the presence of the polyamine, suggesting that the apparent enhancement may not be sufficient to produce bone formation *in vivo*.

c. Transduced peripheral blood cells will then be tested *in vitro* with a heterotopic bone assay to verify their ability to induce bone formation *in vivo*. (months 8-12).

We next tested whether the purified peripheral blood mononuclear cells (10^7 cells) transduced with Ad5F35BMP2 (5000 vp/cell) in the presence of GeneJammer® could produce heterotopic bone when delivered to a mouse quadriceps muscle. Briefly, the cells were isolated, transduced as described in the previous section and cultured for 24 hours prior to injection into the mouse. Cell viability was examined prior to injection and found to be 95%. The quadriceps muscles were analyzed two weeks after the induction, for the presence of bone or cartilage. In all cases, we observed no bone or cartilage formation with the Ad5F35BMP2 transduced human peripheral blood mononuclear cells even with inclusion of GeneJammer® during the transduction, thus confirming that the level of BMP2 expression was not sufficient to induce bone formation.

d. Alternative strategy:

We propose to circumvent this problem by adapting our studies to skin fibroblasts. Skin biopsies can readily be obtained from patients at the time of spine fusion. We propose that these biopsies can then be transduced with our Ad5F35BMP2 virus, and encapsulated with hydrogel, without prior dissociation. Hence this approach would be readily adaptable to the operating room. Therefore we request the approval to pursue

further studies using skin biopsies, isolated from mice. In these studies, we can determine the conditions necessary to achieve sufficient BMP2 production to induce rapid bone formation, and spine fusion.

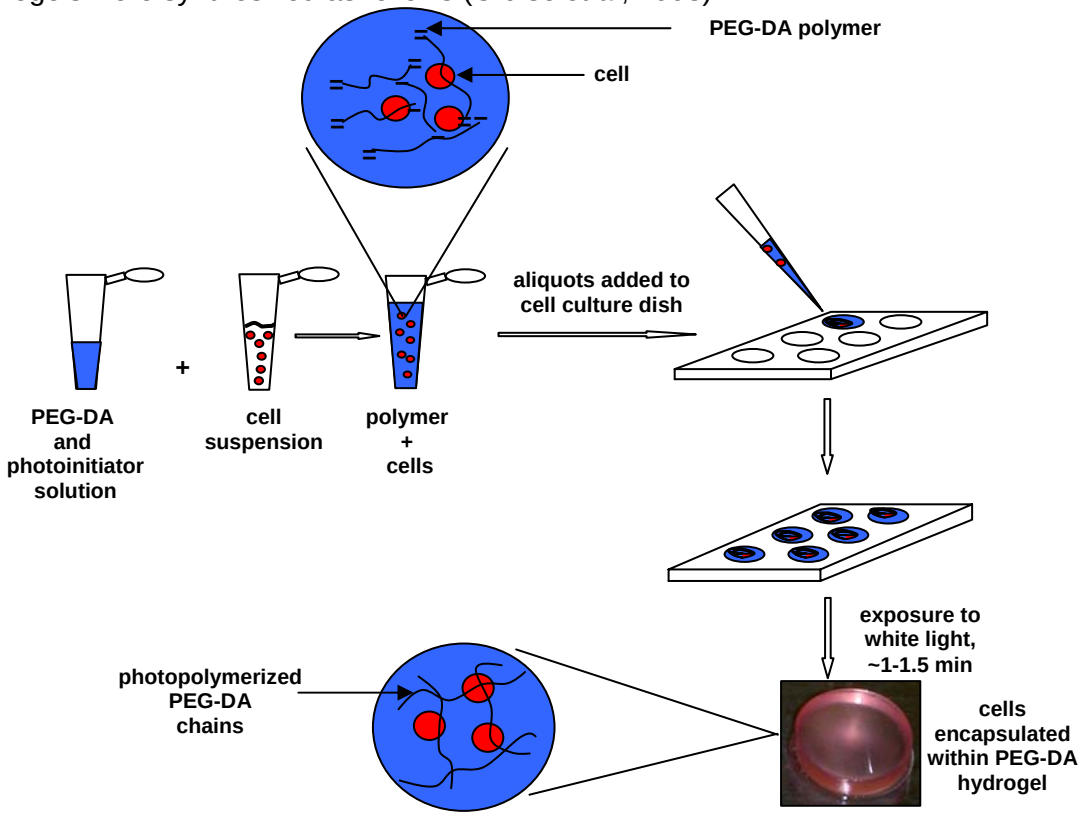
Task 2: To ensure the production of high levels of BMP2 at local fusion sites, by delivering the osteoinductive factor via hydrogel-encapsulated Ad5F35BMP2 transduced peripheral blood cells. (Months 12-26). Development and implementation of hydrogel materials for encapsulation of the Ad5F35BMP2 transduced cells will provide essential safety with the sequestration of the cells, as well as provide ability to direct the location of BMP2 expression, and bone formation. Further, hydrogel encapsulation provides immunoprotection to the transduced cells, potentially extending BMP2 expression as compared with the transduced cells delivered through direct injection that are rapidly cleared by the immune system.

The optimal hydrogel formulation would provide an adequate environment for the transduced cells to both survive and continue to synthesis and secrete the BMP2. Therefore we tested several different molecular weights of the polymer, each having slightly different pore sizes as well as structural properties. Since compression of the material may reduce cell viability as well as disrupt the encapsulation, we tested both properties of the different gel formulations.

a. Perform *in vitro* testing of hydrogels containing Ad5F35BMP2 transduced human peripheral blood. Various hydrogel formulations will be tested to determine the optimal conditioned for BMP2 diffusion. (Months 12-20).

Comparison BMP2 release from PEG-DA hydrogels of varying molecular weight.

Hydrogels were synthesized as follows (Cruise *et al*, 1998).



PEG-DA (6000 Da, 10000 Da, 20000 Da) was synthesized from PEG (Fluka, Milwaukee, WI) as previously described (DeLong SA *et al*, 2005). Covalently immobilized gradients of bFGF were linked to the hydrogel scaffolds for directed cell migration. The dried polymers were then dissolved in 10 mL of ultrapure water and purified by dialysis (MWCO 3500 Da; Fisher Scientific, Pittsburg, PA) against deionized water for 3 days. The purified polymers were then lyophilized and stored at -20 ºC. The hydrogel disks (11.5 mm × 0.5 mm) were

photopolymerized by combining filter sterilized (0.2 µm filter; Gelman Sciences, Ann Arbor, MI) 0.1 g/mL PEG-DA (10 % w/v) with 1.5 % (v/v) triethanolamine/hepes buffered saline (HBS, pH 7.4), 37 mM 1-vinyl-2-pyrrolidinone, 10 mM eosin Y as the initiator, and MRC-5 or MRC-5 cells transduced with Ad5F35-BMP-2 viral vectors (tMRC-5) for hydrogels with cells for a final concentration of 1, 5, or 10 million cells/disk. For characterization of the prepared hydrogels, photopolymerized PEG-DA hydrogels were dried in a vacuum oven for 1 week, after which the dry weights were recorded and then the hydrogels were reswollen in 1X PBS buffer and the swollen weights were recorded. The swelling ratio, water content, molecular weight of the crosslinks, M_c, and the mesh sizes of the prepared hydrogels were then determined. A volume of 150 µL of the reaction solution was pipetted into the wells of a 48-well cell culture dish that was exposed to surgical white light for 2 min. The hydrogels were then transferred to 150 mm × 25 mm cell culture dishes (Corning Inc., Corning, NY) to which 35 mL cell culture media was added. Control cells were plated onto either 75 cm^2 or 225 cm^2 tissue culture flasks (Fisher Scientific; Pittsburg, PA) that were pretreated with filter sterilized 1% gelatin solution (w/v) (Sigma, St. Louis, MO) and maintained in 35 mL of media with the antibiotic-antimycotic.

The molecular weight of the PEG-DA polymers determines the mesh or pore size of the hydrogel, which can impact both diffusion of essential nutrients and oxygen through the materials for cell survival, as well as release of BMP2 for induction of bone. Therefore we tested three different molecular weights; 6 kDa, 10 kDa and 20 kDa of PEG-DA. Table 2 shows the molecular weights (M_c) of the crosslinked PEG-DA and the resulting mesh sizes of the 6kDa, 10 kDa, and 20 kDa hydrogels.

Table 2	Swelling Ratio	Water Content (%)	M_c (g mol^{-1})	Mesh size (Å)
10 % PEG-DA 6 kDa	9 ± 0.7	89 ± 0.7	617 ± 98	34 ± 3
10 % PEG-DA 10 kDa	11 ± 0.4	91 ± 0.3	780 ± 54	39 ± 2
10 % PEG-DA 20 kDa	17 ± 0.2	94 ± 0.1	1556 ± 24	65 ± 1

Varying amounts of BMP2 protein was found to be released from the different hydrogels depending on their molecular weights. As can be seen in Figure 7, BMP2 protein was significantly inhibited by the hydrogel encapsulation as compared to the plated cells, regardless of the molecular weight of the polymer. Encapsulation of 1 million cells within the hydrogels resulted in ~20-fold reduction in BMP-2 protein compared with the controls ($p < 0.001$) and the expression was biphasic over the 15 d period with highest expression observed on day 5. In addition, BMP-2 levels from cells within the different molecular weight hydrogels for each time interval were the same except for days 3 and 9 in which expression from the PEG-DA (20 kDa) hydrogel was higher than both the 6 kDa and 10 kDa hydrogels for day 3 ($p < 0.01$) and expression from PEG-DA (6 kDa) was higher in comparison with PEG-DA 10 kDa and 20 kDa for day 9 ($p < 0.05$). Since the hydrophobic properties of the polymer are not conducive to protein binding, these results are somewhat surprising. One possible explanation is that the BMP2 protein may be forming structures that are much larger than the pore sizes in any of the polymers since even the 20 kDA polymer pore sizes are smaller than many large protein complexes. Alternatively, these numbers may represent cell death, suggesting that only a small percentage of the encapsulated cells are still viable.

We next escalated the cell number per mL of gel to determine if we could increase the amount of BMP2 secreted into the media (Figure 7 A, B, C). We observed a significant increase in BMP2 which correlated with the increase in cell number. We obtained ~600 pg/mL, ~4600 pg/mL, ~8000 pg/mL of BMP2 protein when 1, 5, and 10 million cells respectively, were encapsulated, as determined by ELISA (Figure 7). However, in all cases we observed a ≥60% drop in BMP2 within the media collected from the encapsulated cells versus the plated. Interestingly, the results do not suggesting that the loss of BMP2 is due to overcrowding of the cells within the polymer because we see significantly more BMP2 produced when more cells are added to the same volume of polymer. Since we routinely use 5 million cells to induce bone formation comparison between of BMP2 release between the plated cells (approximately 15,000 pg/mL) and our optimal encapsulation conditions 10 millions cells (approximately 8000), is still significant at 50% drop, but was still sufficient to produce heterotopic bone (see *Task 2 section b*). Further escalation of the cell number above 10 million within this volume of hydrogel, resulted reduced BMP2 expression presumably due to cell death from overcrowding (data not shown).

Figure 7A:

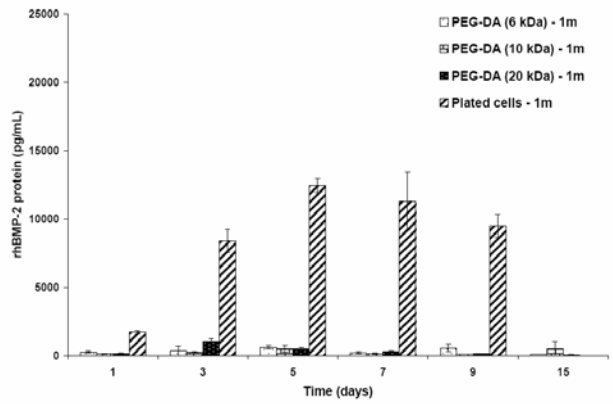


Figure 7B:

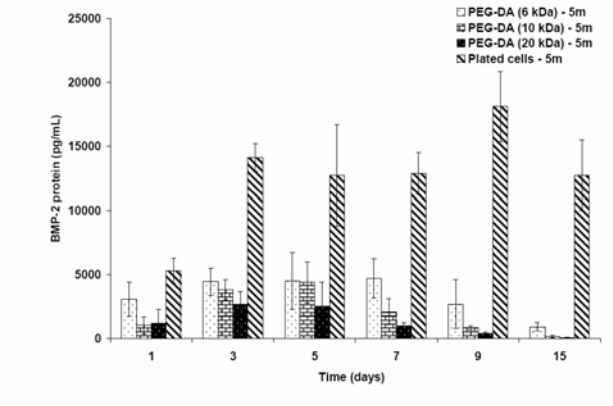


Figure 7C

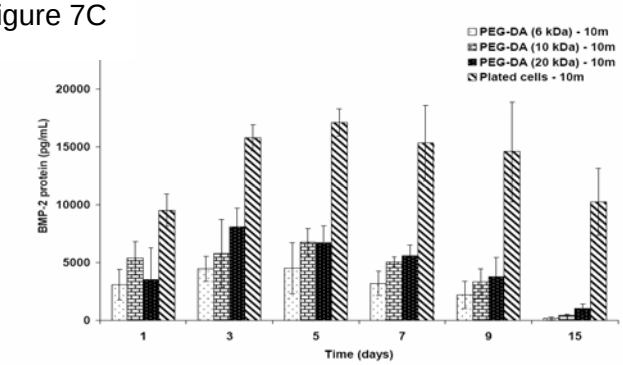


Figure 7: Evaluation of BMP2 expression from Ad5F35BMP2 transduced MRC-5 (tMRC-5) (A) 1 million, (B) 5 million and (C) 10 million cells encapsulated within PEG-DA (6 kDa, 10 kDa, 20 kDa) hydrogels. BMP2 was quantified by ELISA (R&D Systems Inc). Negative hydrogel and plated controls were below the level of detection for this assay. Data reported as mean ± SD, n = 5.

Surprisingly, the expression of BMP2 from the encapsulated cells appears to decrease with time as compared to the cells directly plated, which suggests the conditions for the cells may be suboptimal. Since the cells cannot bind the hydrogel material, perhaps this is a direct response to their lack of adherence, since these are fibroblasts. One method which would circumvent this problem is to introduce binding sites within the hydrogel materials that would provide the cells attachment sites to better enhance their survival and transgene expression. Alternatively, we propose to transduce and encapsulate non-dissociated skin fibroblasts from skin biopsies in future studies, thus they would already possess attachment sights in the encapsulated extracellular matrix that would be encapsulated as well.

Mechanical testing of PEG-DA hydrogels

To assess the compressive strength of the PEG-DA hydrogels after encapsulation with tMRC-5 cells and to select the hydrogel with the best mechanical property that supports the highest gene expression, we performed compressive testing with the PEG-DA hydrogels of various molecular weights containing 10 million tMRC-5 cells. Briefly, PEG-DA (10 kDa and 20 kDa) hydrogel solution with and without 10 million tMRC-5 cells were formulated as described above. The solution was then placed in a rectangular glass mold (~1.4 mm thickness) and exposed to surgical white light for 2 min. The faceplate was removed and a cork borer was used to cut out 11.5 mm diameter disks that corresponded to a final volume of 150 µL. The disks were then transferred to cell culture plates to which 35 mL of complete media was added and the plates were incubated overnight. Prior to analysis, the hydrogels were dabbed with a Kimwipe to remove excess surface media and placed between two parallel platens mounted on an Instron® 3342 (Canton, MA) mechanical tester and the compression modulus of the hydrogels were determined with a 10 N load cell at a crosshead speed of 1 mm/min using the Instron® Series IX software.

The compressive modulus of the PEG-DA hydrogels only was 454 kPa, 395 kPa, and 155 kPa for the 6 kDa, 10 kDa, and 20 kDa hydrogels respectively (Figure 8). Encapsulation of the hydrogels with 10 million

tMRC-5 cells resulted in a decrease in compressive modulus of 153 kPa ($p < 0.001$), 236 kPa ($p < 0.01$), and 90 kPa ($p < 0.05$) for PEG-DA 6 kDa, 10 kDa, and 20 kDa respectively.

Figure 8

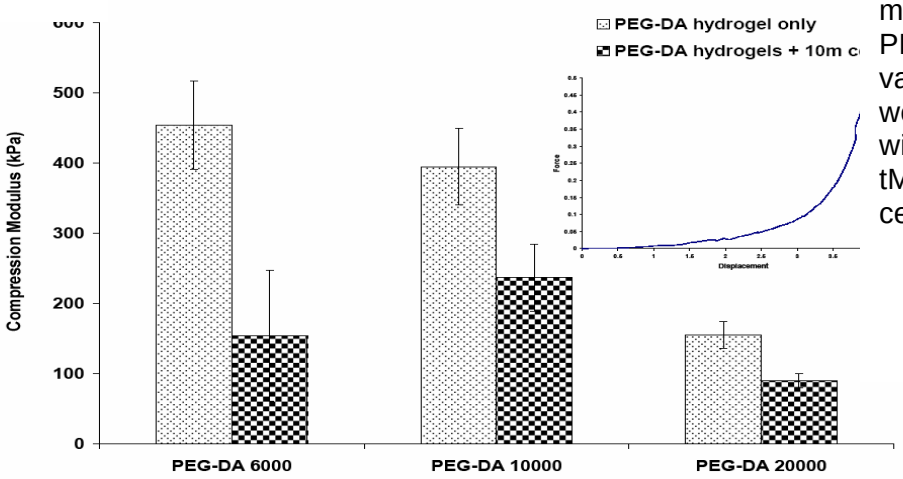


Figure 8: Compression modulus for prepared PEG-DA hydrogels of varying molecular weights with and without 10 million tMRC-5 encapsulated cells after 24 h.

Cell viability of encapsulated cells

The cell viability of MRC-5 and tMRC-5 cells was determined after encapsulation in PEG-DA (10 kDa) hydrogels after 1 and 7 days according to manufacturer's specifications using a LIVE/DEAD® Viability/Cytotoxicity (Molecular Probes; Eugene, OR) kit. Briefly, hydrogel disks with 10 million MRC-5 and tMRC-5 cells were formulated and photopolymerized in 48-well cell culture plates as described above. The hydrogels were then transferred to cell culture dishes to which 35 mL of media was added. On days 1 and 7, the media was removed and the hydrogels were transferred to 6-well plates. The disks were washed with sterile tissue culture grade phosphate buffered saline (PBS) (1X) three times for 10 min each. The fluorophore solution was then prepared by adding 20 µL ethidium homodimer (2 mM) and 5 µL calcein AM (4 mM) to 10 mL 1X PBS. The resulting solution was vortexed and 2 mL was then added to each well containing the hydrogels. The plate was incubated for 45 min at 37 ºC after which the hydrogels were washed as previously described (Cruise *et al* 1998) and the fluorescence of the live and dead cells were analyzed using a Zeiss LSM 510 confocal microscope (Thornwood, NY).

Results suggest little change in cell viability in either population between 1 and 7 days in culture (Table 3).

Table 3: Results of Cell Viability	**24 hours**		**7 days**	
	Live Cells	**Dead Cells**	**Live Cells**	**Dead Cells**
tMRC	71% ± 9%	29% ± 9%	69% ± 8%	31% ± 8%
encapMRC	58% ± 7%	42% ± 7%	60% ± 14%	40% ± 14%

The data suggests that the cells are surviving for at least one week, although this does not rule out that cell death is responsible for the drop in BMP2 expression at two weeks. We propose to further analyze cell viability at longer time points, and compare the results to those when cellular binding sights are engineered into the material. We also propose to measure and compare the release of BMP2 the non-dissociated skin fibroblasts in the presence of and absence of hydrogel encapsulation.

Characterization of secreted BMP-2

In order to verify that the secreted BMP-2 protein from the PEG-DA hydrogels was present, functional, and intact, alkaline phosphatase activity was investigated in W20-17 cells and Western blot analysis was performed with conditioned media from cultured tMRC-5 cells and PEG-DA hydrogels with 10 million tMRC-5 cells. Results from the Western blot analysis showed that not only was the secreted BMP-2 protein from the hydrogels the same as the secreted BMP-2 from the plated controls but that only cells transduced with the

Ad5F35-BMP2 produced detectable BMP-2 (Figure 9A). The results from the alkaline phosphatase data showed that the conditioned media from the hydrogels produced similar levels of alkaline phosphatase activity as the media from the plated cells except for day 1 ($p < 0.001$) (Figure 9B).

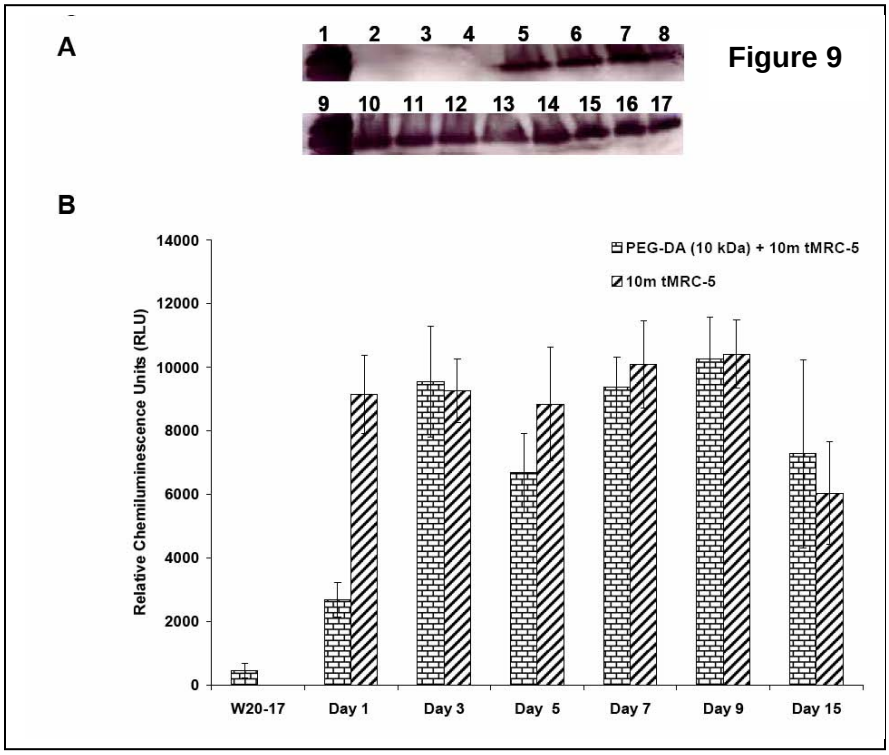


Figure 9: Detection of BMP2 protein and activity. (A) Western blot analysis for the detection of BMP-2 protein. Human recombinant BMP2 (lane 1), conditioned medium from PEG-DA (10 kDa) hydrogels only (lane 2), conditioned medium from 10 million MRC-5 cells encapsulated within PEG-DA (10 kDa) hydrogels (lanes 3 and 4), conditioned medium from 10 million tMRC-5 cells control (lanes 5-8), human recombinant BMP2 (lane 9) conditioned medium from 10 million tMRC-5 cells encapsulated within PEG-DA (kDa) hydrogels (lanes 10-17). **(B)** Alkaline phosphatase activity in W20-17 cells after addition of conditioned media from PEG-DA (10 kDa) hydrogels with 10 million tMRC-5 cells and control plated tMRC-5 cells. Data reported as mean ± SD, n = 5.

b. Perform *in vivo* testing of hydrogels containing Ad5F35BMP2 transduced human peripheral blood in heterotopic bone formation. Radiological and histological analysis will be done to confirm that the resultant bone formation is local rather than progressive (Month 20-26).

Optimization of hydrogel shape for in vivo implantation

In our first preliminary experiments, the 150 uL hydrogel disks were implanted into the rear quadriceps muscle in a mouse and endochondral bone formation was allowed to progress for two weeks prior to analysis. Although in these experiments we did observe bone formation, however due to their large size we experienced difficulty in maintaining the integrity of the hydrogel during implantation, and the large volume left little room in the tissues for extensive bone formation. Hence we developed an alternative structure for the hydrogel material, which utilized the same number of cells, but were in smaller "bead-like" structures that were formed along a surgical thread which allowed for easier transfer into the mice. However prior to implantation we confirmed that the new structure had not significantly altered either BMP2 release (Figure 10), structural integrity, or cell viability (data not shown).

The results of the ELISA data for the polymerized "beads-on-a-string" showed that the plated tMRC-5 controls produced ~2.5-fold higher BMP-2 levels as compared with both the hydrogel beads and disks (Figure 10). However, the tMRC-5 cells within the hydrogel beads produced peak levels of ~7600 pg/mL BMP-2 on day 5 as opposed to ~6800 pg/mL ($p>0.005$) from the tMRC-5 cells within the hydrogel disks with a similar biphasic profile that was previously observed for the hydrogel disks. This slight increase in BMP-2 levels for the hydrogel beads as compared with the disks (~800 pg/mL difference) was maintained over the 15 day period except for day 1 ($p<0.01$) (~4000 pg/mL difference).

25 uL beads

150 uL disk

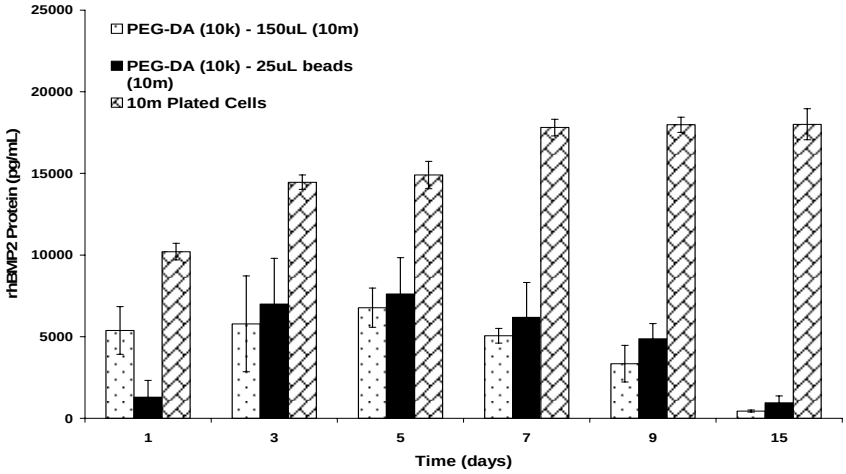


Figure 10: Comparison of gene expression from 10 million adenovirus transduced MRC-5 cells (tMRC-5) in PEG-DA (10 kDa) hydrogels disks with 10 million tMRC-5 cells in PEG-DA (10 kDa) hydrogels beads with ELISA assay. Negative hydrogel and plated controls were less than 100 pg/ mL BMP-2 detected. Data reported as mean ± SD, n = 5

Micro-CT analysis of bone

To determine the amount of ectopic bone formed in NOD/SCID mice with implanted PEG-DA hydrogels containing tMRC-5 cells, the volume of mineralized ectopic tissue in the muscle was evaluated.

Approximately three weeks after implantation, the rear hind limbs NOD/SCID mice were scanned at 14 µm resolution with a commercial micro-CT system (GE Locus SP, GE Heathcare, London, Ontario). Three-dimensional reconstructions of the lower limb bones and any mineralized tissue in the surrounding muscle were created at 29 µm resolution to visualize ectopic mineralized tissues. A complex volume of interest was defined for each specimen to include only the ectopic mineralized tissue, and a threshold was chosen to exclude any non-mineralized tissue. The total volume of ectopic bone was then measured (eXplore MicroView, v. 2.0, GE Healthcare, London, Ontario).

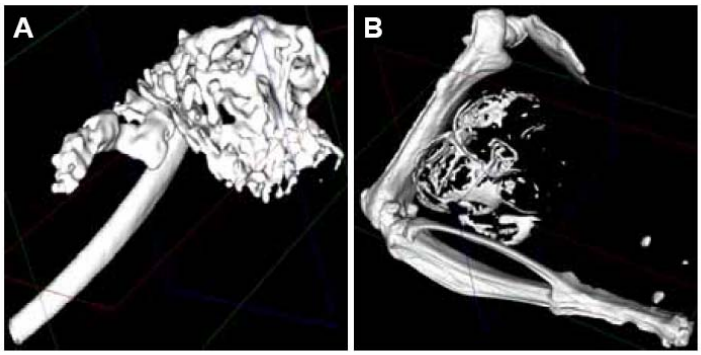


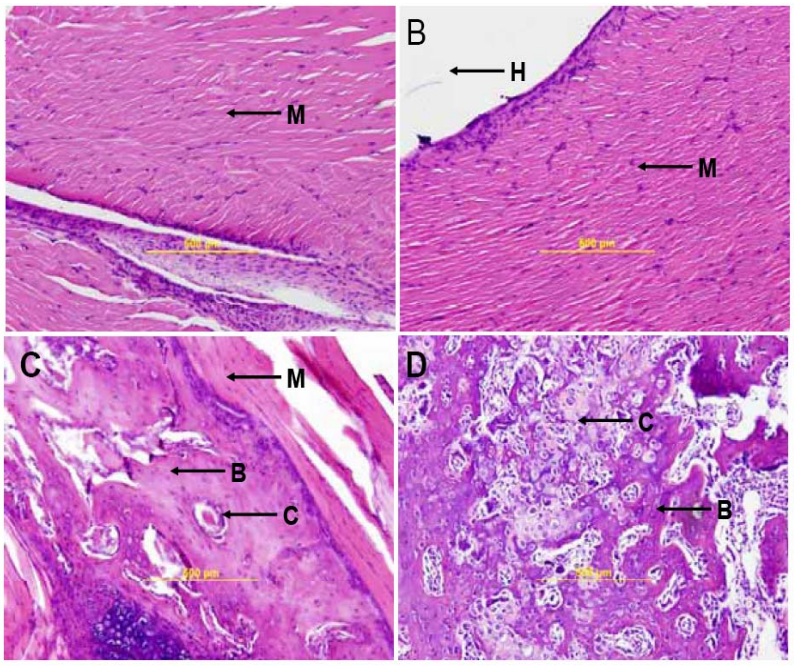
Figure 11: Micro-CT analysis of ectopic bone formation at 3 weeks. (A) bone formation from 10 million tMRC-5 cells administered via intramuscular (i.m.) injection and (B) bone formation from PEG-DA (10 kDa) constructs with 10 million tMRC-5 cells following surgical implantation.

The mean amount of mineralized tissue in the muscle of the mice was 31.8 ± 7.8 mm^3 and 39.5 ± 5.0 mm^3 for mice injected with tMRC-5 cells only and mice implanted with the PEG-DA hydrogels encapsulated with tMRC-5 cells respectively (Figure 11). There was no ectopic bone detected in the mice that were administered untransduced MRC-5 cells or those transduced with Ad5F35-HM4 viral vectors. Surprisingly, the volume of mineralized tissue between the samples was approximately equivalent. Presumably this was due in part to the fact that all the beads had bone surrounding them whereas in delivery of the cells directly, bone formed only around the area of the initial injection. This finding is significant in that the animals receiving the injected cells received 50-60% more BMP2 than those that got the hydrogel beads suggesting that placement of the BMP2 may be as critical to inducing robust bone formation as a threshold amount. Further this finding suggests that the bone reaction can only occur within a limited distance from the BMP2 itself even in these systems with sustained expression.

We are currently repeating the study in C57BL/6 (immunocompetent) mice. Early radiological analysis suggests similar results to those obtained with the Nod/Scid. In these studies we encapsulated a fibroblast cell line derived from C57BL/6 mice that had been transduced with Ad5BMP2 in the presence of GeneJammer. We have developed a protocol in which we can obtain the same level of BMP2 expression as that obtained when we transduce human fibroblasts with Ad5F35BMP2. From studies discussed in our first progress report, we have determined that the presence of a complete immune system does not alter the stages or timing of endochondral bone formation in this assay. A complete report of this data will be presented at the up coming Gene Therapy Meeting in Baltimore MD.

Histological analysis

To demonstrate the physiology of the ectopic bone formed in the muscle as well as to assess the biocompatibility of the PEG-DA hydrogels as a result of surgical implantation, sections of the muscle tissue was stained with hematoxylin and eosin. The stained sections showed that there was bone as well as cartilage formed in the muscles of the mice implanted with the hydrogels that was comparable to injected tMRC-5 transduced cells only. In addition, there were little or no immunological reactions due to the presence of the PEG-DA hydrogels as shown from the negative hydrogel control, whereas there were noticeable amounts of recruited cells in the area of the injection site (Figure 12).



In addition, the cell viability of tMRC-5 cells encapsulated within PEG-DA (10 kDa) hydrogels two weeks after surgical implantation was evaluated. The LIVE/DEAD staining of the cells showed that 72% ± 22% and 28% ± 22% of the cells were live and dead respectively in the left leg. This data was consistent with the right leg that had 75% ± 3% and 25% ± 23% live and dead cells respectively.

Figure12: Histological evaluation of ectopic bone formation at 3 weeks. (A) 10 million MRC-5 cells transduced with HM4-1; (B) PEG-DA (10 kDa) hydrogels with 10 million MRC-5 cells transduced with HM4-1; (C) 10 million tMRC-5 cells; (D) PEG-DA (10 kDa) hydrogels with 10 million tMRC-5 cells.

Task 3: To achieve posterolateral spine fusion by percutaneous injection of the encapsulated Ad5F35BMP2 transduced human peripheral blood cells, into the paraspinous musculature of rats. (Months 24-36).

- **a.** Confirm spine fusion using Ad4F35BMP2 transduced human peripheral blood cells encapsulated in hydrogels using an athymic rat model for spine fusion. Spine fusion in the rat model will be assessed by both histological and radiological analysis. (Months 24-30).
- **b.** Compare resultant spine fusion using the encapsulated transduced cells in both immuno-incompetent and immunocompetnet rats. (Months30-36).
- **c.** Approximately 150 rats (Sprague Dawley and NIH nude rats) will be used to complete the experiments in this task. Human peripheral blood will be collected from no more than 20 individuals, and approximately 6 mls/individual.

The experiments described in ***Task 3*** are slated to be completed in this upcoming year.

Key Research Accomplishments

- We have developed a novel non-receptor mediated delivery system for adenovirus, to provide for higher level transduction efficiency.

- We have developed a formulation of hydrogel that provides for sufficient BMP2 expression to induce *in vivo* bone formation. In experiments in which one large 150 ul disk was implanted, we had a very thin shell of bone surrounding the material. To circumvent this we went ahead and pre-formed the hydrogel encapsulated cells into 25 uL bead structures. These were then implanted thus allowing more room for bone formation to occur between the beads. Micro-CT analysis of the bone formation 2 and 3 weeks after injection showed significant amounts of mineralization that was confirmed to be mature bone via histology.
- The area of new bone formation as determined by microCT was equivalent between the animals receiving the hydrogel beads as compared to those that received the direct injection of cells. Presumably this was due in part to the fact that all the beads had bone surrounding them whereas in the direct injection, bone formed only around the area of the initial injection. This finding is significant in that the animals receiving the injected cells received 50-60% more BMP2 than those that got the hydrogel beads suggesting that placement of the BMP2 may be as critical to inducing robust bone formation as a threshold amount. Further this finding suggests that the bone reaction can only occur within a limited distance from the BMP2 itself even in these systems with sustained expression.
- Early preliminary data using C57BL/6 (immunocompetent) mice suggests similar results to those obtained with the NOD/Scid. In these studies we encapsulated a fibroblast cell line derived from C57BL/6 mice that had been transduced with Ad5BMP2 in the presence of GeneJammer. We have developed a protocol in which we can obtain the same level of BMP2 expression as that obtained when we transduce human fibroblasts with Ad5F35BMP2.
- We propose in the next year to initiate studies not only to enhance the bone formation assay, by incorporation of VEGF-D but also to add into the beads specific sites that can be recognized by osteoclasts that can eventually remodel the material into normal skeletal bone. Further, we propose to introduce another adenovirus vector that is turned on in the presence of tetracycline, but off in the absence. This vector will allow us to turn off BMP2 expression during the period by which osteoclasts start to remodel the hydrogel material.

Reportable Outcomes:

Abstracts:

1. Hydrogel Encapsulation of Adenovirus-transduced Cells Expressing BMP2 for local osteoinduction. Malavosklish Bikram, Christine Dilling, André M. Gobin, Elizabeth A. Olmsted-Davis, Alan R. Davis and Jennifer L. West. American Society of Gene Therapy 8th Annual Meeting, St Louis Missouri 2005.
2. Novel Compound Enables Transduction in the Absence of an Adenovirus-Specific Receptor. Christine M. Fouletier-Dilling, Pablo Bosh, Alan R. Davis, Jessica A. Shafer, Steven L. Stice, Zbigniew Gugala, Francis H. Gannon, and Elizabeth A. Olmsted-Davis. American Society of Gene Therapy 8th Annual Meeting, St. Louis Missouri 2005.
3. BMP2 Induced Bone Formation Follows Similar Cellular Events in the Presence and Absence of an Immune System. Christine M. Fouletier-Dilling, Zawaunyka Lazard, Jessica A. Shafer, Francis H. Gannon, Alan R. Davis and Elizabeth A. Olmsted-Davis. American Society of Gene Therapy 9th Annual Meeting, Baltimore MD 2006.

Manuscripts:

1. Christine Fouletier-Dilling, Pablo Bosh, Alan R. Davis, Jessica Shafer, Steven Stice, Zbigniew Gugala, Francis H. Gannon, and Elizabeth A. Olmsted-Davis (2005). A Novel Compound Enables High-Level Adenovirus Transduction in the Absence of an Adenovirus-Specific Receptor. Human Gene Therapy 16(11): 1287-1298.
2. Malavosklish Bikram, Christine Fouletier-Dilling, Jessica A. Shafer, John A. Hipp, Alan R. Davis, Elizabeth A. Olmsted-Davis and Jennifer L. West. Gene Therapy (In review). Ectopic Bone Formation from Hydrogel Carriers Loaded with BMP2-transduced Cells

Conclusions:

We conclude that the peripheral blood mononuclear cells can be transduced to express BMP2, using the chimeric adenovirus vector Ad5F35, along with the polyamine, GeneJammer®. However, this level of induction coupled to the significant reduction in BMP2 expression from the hydrogel encapsulated cells, is not

sufficient to produce endochondral bone. **We propose the use of skin fibroblasts as a potential alternative. Skin biopsies could readily be obtained from patients with limited risks associated and transduced with Ad5F35BMP2 vectors, encapsulated in hyrdogel, and injected into the fusion sight.** We propose to test this hypothesis by quantifying BMP2 expression from skin biopsies isolated from mice, transduced with BMP2 and either implanted directly into the fusion sight or encapsulated prior to implantation.

We have demonstrated the ability of the BMP2 transduced cells to induce spine fusion in Nod/SCID mice, and have validated that the bone induction mechanism in Nod/SCID, works similarly in immune competent mice. Finally we have demonstrated the ability to encapsulate the BMP2 transduced cells in a hydrogel, and still maintain their viability and efficient secretion of BMP2. These are the first steps in developing a safe and efficacious therapy for the tissue engineering of bone.

However, for this system to become a reality, we must demonstrate the ability of the Ad5F35BMP2 transduce skin fibroblasts, encapsulate in hydrogel, and implanted through injection into the musculature surrounding the spine in immune competent mice, to induce spine fusion. Further we must confirm that the cells remain inside the hydrogel and localized to the injection site. To these ends we plan to initiate experiments to implant both hydrogel encapsulated beads near the spine and further characterize the resultant bone formation as well as perform cell tracking studies to demonstrate both viability as well as sequestration. These experiments will be initiated within the next few months.

An additional obstacle to overcome is the level of BMP2 secretion after the cells are encapsulated in hydrogel. If the reduction in BMP2 activity is due to lack of cell adherents to the hydrogel, hence cells are not as capable of protein synthesis, the skin biopsies should improve this, since the lack of cell dissociation would allow the cells to stay adhered in there normal structural environment. Finally we propose to enhance the bone formation assay by inclusion of a secondary gel which will enhance vascular stem cell trafficking and hence necessary precursors for cartilage and bone formation.

Completion of this project would advance the current state of gene therapy in this field by eliminating the search for an optimal osteoprogenitor cell and scaffolding. Posterolateral spine fusion, which normally results in 500-1000 cc of blood loss as well as a 5 to 7 day hospital stay and a recovery period of up to a year, could be performed on an outpatient basis with this minimally invasive procedure, without concern over undue morbidity. This technology would benefit a broad age range of patients, and greatly reduce treatment costs as well as loss work time. Our proposed method has the potential to improve the safety of current spine surgery techniques and would offer an alterative to patients who require spine fusion but are not candidates for major surgery.

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Appendices:

HUMAN GENE THERAPY 16:1287–1297 (November 2005)

Novel Compound Enables High-Level Adenovirus Transduction in the Absence of an Adenovirus-Specific Receptor

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ABSTRACT

Viral vectors are extensively used to deliver foreign DNA to cells for applications ranging from basic research to potential clinical therapies. A limiting step in this process is virus uptake and internalization into the target cells, which is mediated by membrane receptors. Although it is possible to modify viral capsid proteins to target the viruses, such procedures are complex and often unsuccessful. Here we present a rapid, inexpensive system for improving transduction of cells, including those that lack receptors for adenovirus fiber proteins. Addition of GeneJammer (Stratagene, La Jolla, CA) during the adenovirus transduction led to a significant increase in both the total number of transduced cells and the level of transgene expression per cell. Studies using cell lines deficient in adenovirus receptors demonstrated that addition of GeneJammer provided a novel cellular entry mechanism for the virus. These findings were tested in a cell-based gene therapy system for the induction of bone, which is contingent on high-level expression of the transgene. Inclusion of GeneJammer in either Ad5BMP2 or Ad5F35BMP2 transduction of a variety of cells demonstrated a correlating increase in bone formation. The results suggest a novel and versatile method for achieving high-level transduction using adenovirus.

OVERVIEW SUMMARY

Adenovirus vectors are useful in gene transfer, ranking second behind DNA transfection as a DNA delivery method. However, host range and tissue tropism restrict the use of specific adenovirus vectors, sometimes necessitating the time-consuming construction of new vectors with more appropriate cell specificity. This is particularly important in bone formation using Ad5BMP2, where inefficient transduction can result in the total lack of bone formation. We describe here a novel methodology, using GeneJammer, that increases the transduction efficiency of adenovirus vectors in general in a receptor-independent fashion. We show that the use of this compound enables bone to be formed readily *in vivo* in immunocompetent animals.

INTRODUCTION

OSTEOINDUCTIVE CYTOKINES comprise the bulk of known osteoinductive agents as a class of relatively small secreted molecules that are capable of promoting the formation of bone. Some of the most extensively studied of these factors are the bone morphogenetic proteins (BMPs), which are part of the transforming growth factor (TGF)-β superfamily and were originally isolated on the basis of their ability to induce ectopic bone. Turgeman *et al*. (2002) suggested that BMP2 may also be capable of enhancing bone formation by adult mesenchymal stem cells, providing a possible role in treatment of osteoporosis. The recombinant human proteins BMP2 and BMP7 (rhBMP2 and rhBMP7) have been approved for restricted clinical use; however, large doses of these recombinant proteins are

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required to induce adequate bone repair, suggesting that the mode of BMP delivery still requires further optimization. Furthermore, BMP-induced osteogenesis, even under ideal conditions, does not approach the efficiency of bone formation seen during normal fracture repair (Franceschi *et al*., 2004). Also, delivery of these proteins requires a carrier such as collagen sponge carriers (Bonadio *et al*., 1999), which can also elicit responses that often influence the bone formation process.

Alternatively, gene therapy approaches have been developed to circumvent the problems associated with delivering the protein itself (Musgrave *et al*., 2000; Olmsted-Davis *et al*., 2002). Studies using both nonviral and viral delivery systems have successfully induced bone formation in several animal models. Park *et al*. (2003) used liposomes to transfect mesenchymal stem cells to heal mandibular defects in rats by an *ex vivo* strategy that showed markers of new bone matrix such as osteopontin and osteocalcin within 2 weeks of gene transfer. Musgrave *et al*. (2000) have shown that primary bone marrow mesenchymal stem cells (hBM-MSCs) transduced with Ad5BMP2 produced no radiographic ossification. We demonstrated (Olmsted *et al*., 2001) that adenovirus serotype 5 (Ad5) elicits only minimal amounts of BMP2 synthesis on transduction of hBM-MSCs because of the lack of adenovirus-specific receptors. Further, we previously demonstrated a critical link between the amount of bone morphogenetic protein-2 expressed in the tissue and the production of bone (Gugala *et al*., 2003). Therefore the poor efficiency of delivery and transient expression of the gene are significant challenges that need to be overcome for these strategies to be truly effective.

Consequently, several approaches have been undertaken in order to circumvent these problems. A limiting step in adenoviral uptake is indeed the entry into target cells, which is mediated by the coxsackievirus–adenovirus receptor (CAR) and cellular $\alpha_v\beta$ integrins. In many cell types, these receptors are either absent or expressed at low levels, leading to poor transduction efficiency by adenovirus. For instance, CAR expression is highly variable and most stem cells and fibroblast-type cells are poorly infected by adenoviral vectors because of low expression of CAR (Hidaka *et al*., 1999; Sakurai *et al*., 2003).

Investigators have attempted to circumvent this problem by introducing genetic fiber modifications in Ad, which provides an alternative cellular entry route. Chimeric Ad5 vectors possessing fiber proteins derived from subgroup B Ad serotypes such as Ad35 have become increasingly popular as gene transfer vectors because they can efficiently deliver genes to cell types that are refractory to Ad5 infection (Mizuguchi and Hayakawa, 2002; Olmsted-Davis *et al*., 2002; Gao *et al*., 2003). Group B adenoviruses have been shown to use CD46 as a primary attachment receptor (Gaggar *et al*., 2003; Segerman *et al*., 2003). However, receptors for these chimeric viruses appear to be absent on most murine cells (Mallam *et al*., 2004), which reduces their versatility, and high titers of the purified fiber-modified viruses are difficult to produce.

Polycations and cationic lipids, which form complexes with adenoviral particles, have also been used to facilitate *in vitro* transduction of a range of cell types (Lanuti *et al*., 1999; Toyoda *et al*., 2001). Similarly, adenoviral infection of primitive human hematopoietic cells can be strongly enhanced by several cationic lipids (Harrison *et al*., 1995; Byk *et al*., 1998; Marit *et al*., 2000). Others have used bilamellar cationic liposomes to protect adenovectors from preexisting humoral immune responses (Yotnda *et al*., 2002).

The aim of this study was to investigate the role of a polyamine in enhancement of adenoviral transduction of several cell types *in vitro*, using a proprietary formulation of polyamine and other components in 80% ethanol (GeneJammer; Stratagene, La Jolla, CA). We show a significant increase in both the total number of transduced cells and the level of transgene expression per cell, when the transduction is carried out in the presence of GeneJammer. Studies using cell lines deficient in adenovirus receptors demonstrated that addition of GeneJammer provided a novel cellular entry mechanism for the virus. We further tested this methodology in our cell-based gene therapy system for induction of bone formation. The compound increased cell transduction and achieved a high level of functional BMP2 protein expression, in both murine and human cell types. Further, this increase in BMP2 production correlated with increased bone formation *in vivo*. This finding greatly advances our goal of developing a cell-based gene therapy system for the induction of bone formation. GeneJammer transfection reagent markedly increases the efficiency of adenovirus-mediated gene transfer in cells deficient in adenovirus receptors through an alternative pathway for virus entry into the cell. Hence this methodology provides a rapid, inexpensive technique for transduction of adenovirus-refractive cells.

MATERIALS AND METHODS

Cell culture

Cell lines. Chinese hamster ovary (CHO) cells, a human lung carcinoma cell line (A549), and a murine osteoblast cell line (MC3T3-E1) were obtained from American Type Culture Collection (Manassas, VA). Human lung carcinoma cells (A549) were propagated in Dulbecco's modified Eagle's medium (DMEM; Cambrex Bio Science Walkersville, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml; Invitrogen, Gaithersburg, MD); CHO cells and MC3T3-E1 cells were propagated in RPMI and α-MEM, respectively, supplemented as described above.

The W20-17 mouse stromal cell line (a gift from Genetics Institute, Cambridge, MA) was propagated as described by Thies *et al*. (1992). Briefly, the cells were grown in DMEM supplemented as described above and cultured at a subconfluent density in order to maintain the phenotype. All cell types were grown at 37°C and 5% CO_2 in humidified air.

Human bone marrow mesenchymal stem cells. Discarded human bone marrow products were obtained from healthy donors in compliance with all state and federal regulations, with institutional review board approval. Mononuclear cells were isolated by gradient density centrifugation on Ficoll-Paque PLUS (Amersham Biosciences/GE Healthcare, Piscataway, NJ) and washed twice with Dulbecco's phosphate-buffered saline (PBS; Invitrogen) before culturing. Cells were plated at a density of 5×10^6 cells/cm^2 (Jaiswal *et al*., 1997) in DMEM supplemented with 10% FBS and antibiotics–antimycotics as de-

scribed above. Early adherent fibroblastic cells appeared within 2 days of culture, and after 1 week dead cells and debris were removed by washing with PBS and cells were passaged before confluence. Several vials of these cells were frozen in Origen dimethyl sulfoxide freeze medium (Igen International, Gaithersburg, MD). Where indicated, hBM-MSCs were treated by addition of 1 μM dexamethasone (Sigma, St. Louis, MO) to the culture medium.

Transduction of cells with adenovirus in the presence or absence of GeneJammer

Adenoviruses. Replication-defective E1- and E3-deleted first-generation human Ad5 and/or modified forms in which the normal fiber protein has been substituted for the human adenovirus type 35 fiber (Ad5F35) were constructed to carry cDNAs for either BMP2 or green fluorescent protein (GFP) in the E1 region of the virus (Olmsted *et al*., 2001). Ratios of virus particles (VP) to plaque-forming units (PFU) were 55, 76, 8, 132, and 200 for Ad5BMP2, Ad5F35BMP2, Ad5eGFP, Ad5F35eGFP, and Ad5-empty, respectively, and all viruses were shown to be negative for replication-competent adenovirus. The VP:PFU ratio corresponds to MOIs (plaque-forming units per cell) of 45, 32, 312, 20, of 12.5, respectively.

Cell transduction. Adherent cells (1×10^6) were transduced with adenovirus at three different virus concentrations (2500, 5000, and 10,000 VP/cell) with or without 1.2% GeneJammer. This concentration of the polyamine compound was optimized on the basis of percentage of GFP transduction (data not shown). Briefly, 15 μl of GeneJammer or PBS was added to 500 μl of DMEM without supplements and incubated for 10 min at room temperature. The virus was then added at one of the indicated concentrations and the mixture was further incubated for 10 min at room temperature. This virus–GeneJammer mixture was added to approximately 1×10^6 cells along with 750 μl of DMEM supplemented with 10% FBS and antibiotics–antimycotics. The cells were incubated at 37°C for 4 hr and then the mixture was diluted with 3 ml of fresh medium containing FBS.

Flow cytometry

Transduced cells were trypsinized, washed, and resuspended in PBS 48 hr after the initial transduction. Dead cells and debris were excluded from analysis by inclusion of propidium iodide (PI). Flow cytometric analysis was performed with a FACSCalibur cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA). The percentage of GFP-positive cells was quantified with CellQuest software (BD Biosciences Immunocytometry Systems), using a minimum of 10^5 cells per sample ($n = 3$). Relative fluorescence intensity (RFI) of the GFP-positive cell population was also determined for each sample. All data were taken in triplicate and reported as means and standard deviation. Significance was determined between samples transduced with and without GeneJammer ($n = 3$), using a Student t test with 95% confidence interval ($p < 0.05$) (InStat software; GraphPad Software, San Diego, CA).

A549 cells, hBM-MSCs, and CHO cells were analyzed for CAR, α_v integrin, and CD46 expression. Briefly, the cells at a concentration of 1×10^6/ml were incubated with the primary antibody: CAR, a mouse anti-human antibody (ab9891; Abcam, Cambridge, UK), α_v integrin, a mouse anti-human antibody (MAB1953Z; Chemicon International, Temecula, CA), or CD46, a mouse anti-human antibody (CBL488; Chemicon International) for 15 min on ice. The secondary antibody, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse (553354; BD Biosciences Pharmingen, San Diego, CA) was diluted 1:200 and cells were incubated with the secondary antibody for 15 min on ice. The cell pellet was washed in PBS and the samples were analyzed by flow cytometry.

Quantification of BMP2

BMP2 protein was measured in culture supernatant taken from cells 72 hr after transduction with various concentrations of Ad5BMP2 in the presence or absence of GeneJammer. Briefly, 10^6 cells were transduced as described above and culture supernatant was collected and assayed with a Quantikine BMP2 immunoassay (DBP200; R&D Systems, Minneapolis, MN).

Alkaline phosphatase assay

W20-17 cells were assayed for alkaline phosphatase activity 3 days after addition of either Ad5BMP2, Ad5-empty, or medium, using a chemiluminescence procedure (Olmsted *et al*., 2001). Cellular alkaline phosphatase was extracted by three freeze–thaw cycles in a 100-μl/cm^2 concentration of 25 mM Tris-HCl (pH 8.0) and 0.5% Triton X-100 and activity was then measured by addition of CSPD substrate with Sapphire-II enhancer (Applied Biosystems, Foster City, CA) to the samples. The light output from each sample was integrated for 10 sec (after a 2-sec delay) with a luminometer (TD-20/20; Turner Designs, Sunnyvale, CA). Alkaline phosphatase levels were recorded as relative luminescence units (RLU) and normalized to protein content with the bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL), using bovine serum albumin to derive a standard curve. Data are presented as percent induction relative to that of unstimulated basal control cells. Statistical analysis was performed as described previously. Briefly, all data were taken in triplicate and reported as means and standard deviation. A Student t test with 95% confidence interval ($p < 0.05$) was done between the untreated control and each experimental condition.

Heterotopic bone assay

hBM-MSCs were transduced with Ad5BMP2 (2500 VP/cell) or Ad5-empty (2500 VP/cell), the latter being a control adenovirus type 5 vector that lacks a transgene in the E1-deleted region. Briefly, cells were removed with trypsin, resuspended at a concentration of 5×10^6 cells/100 μl of PBS, and then injected into the hindlimb quadriceps muscle of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (two animals per group). All animals were killed 2 weeks after injection and the hindlimbs were harvested, the skin removed, and the limbs placed in formalin. Bone formation was analyzed by X-ray analysis, using an MX-20 specimen radiography system model (83 sec at 31 kV; Faxitron, Wheeling, IL). All animal studies were performed in accordance with standards of the Department of Comparative Medicine of Baylor College

of Medicine (Houston, TX), after review and approval of the protocol by the Animal Use and Care Committee.

RESULTS

Enhanced viral transduction in the presence of GeneJammer

Adenovirus transduction of coxackie–adenovirus receptor (CAR)-negative cell lines is extremely inefficient, requiring large amounts of virus and resulting in low-level expression of the desired transgene. To enhance virus uptake into the cells, we tested the ability of the commercially available polyamine complex, GeneJammer, to enhance virus transduction. Accordingly, CAR-negative hBM-MSCs (10^6 cells) were transduced with Ad5eGFP virus at three different concentrations (2500, 5000, and 10,000 VP/cell) in the presence or absence of GeneJammer (Fig. 1A). Adenovirus type 5 lacking any transgene (Ad-empty) was used as a negative control. As seen in Fig. 1A, the presence of the polyamine GeneJammer increases the number of transduced hBM-MSCs at all concentrations of virus. The lowest MOI, 2500 VP/cell, resulted in the most dramatic increase in number of transduced cells, with 15% in the absence of GeneJammer and 95% transduced in the presence of the polyamine. Further, we observed an increase in the number of transduced cells with a corresponding increase in virus MOI in the absence of GeneJammer; however, in the presence GeneJammer, the maximum number of transduced cells (95–100%) was found for all virus concentrations.

Because multiple virus particles can enter the same cell, we also analyzed samples transduced in the presence of GeneJammer, and in which we obtained 95–100% cell transduction, to determine whether the intensity of GFP expression increased with virus concentration. As can be seen in Fig. 1B, the intensity of GFP expression from cells transduced with GeneJammer increased with virus dose. Because 100% of the cells were transduced at all virus doses in this population, the increase in GFP intensity presumably represents an increase in the number of virus particles each cell is taking up. The data suggest that GeneJammer not only enhances the number of cells taking up the virus (Fig. 1A) but also the total amount of virus entering any given cell (Fig. 1B).

GeneJammer compound allows adenovirus to enter cells lacking the receptor for fiber

Two potential models exist as to the mechanism by which the polyamine enhances virus uptake. First, GeneJammer may aid in virus binding to its receptor, therefore potentially acting as a coreceptor for the virus internalization. Alternatively, this compound may bind to the virus and promote a novel entry route into the cell. To determine which of these is most likely, we compared the transduction efficiency of various cell types known to have differential expression levels of CAR and α_v integrin (Ad5) or CD46 (Ad5F35), in the presence or absence of GeneJammer. The three cells lines chosen have the following receptor characteristics: A549 cells express high levels of CAR, α_v integrin, and CD46; hBM-MSCs lack CAR but express α_v integrin, and express moderate levels of CD46; and CHO cells express few or no adenovirus receptors (Table 1). As expected, Ad5eGFP (2500 VP/cell) transduced 100% of the receptor-positive A549 cells, whereas less than 15% of the receptor-negative CHO cells were transduced (Fig. 2A and C). hBM-MSCs, which are α_v integrin positive and CAR negative, yielded approximately 10% of cells expressing GFP at this same MOI (Fig. 2B). Parallel transductions done in the presence of GeneJammer showed significant enhancement of transduction in cells lacking CAR but expressing α_v integrin, as well as in receptor-negative cells (Fig. 2B and C). The results in Fig. 2C show that approximately 95% of transduced CHO cells expressed GFP when the adenovirus was delivered in the presence of the polyamine, suggesting that the molecular mechanism is not adenovirus receptor associated but, rather, a novel pathway for virus entry into the cells. We did not see any significant changes between the groups in A549 cells (Fig. 2A) because the cells in the absence of GeneJammer were maximally transduced. However, the data suggest that these two systems for virus entry do not appear to inhibit one another (Fig. 2A).

We next chose to determine whether virus entry into cells via the polyamine was specific to the adenovirus type 5 capsid, and therefore similar experiments were conducted with the altered fiber virus Ad5F35eGFP. Results were similar to those obtained with the Ad5eGFP vector, suggesting a similar mechanism that is not dependent on adenovirus type 5 fiber for entry into the cell (Fig. 2).

BMP2 expression and bone formation in the presence of GeneJammer

We previously reported the inability of Ad5BMP2 to transduce hBM-MSCs, which resulted in low-level expression of BMP2 and lack of bone formation *in vivo* (Olmsted-Davis *et al*., 2002). In addition, because eGFP has been engineered to be extremely stable, whereas BMP2 is significantly less stable at both the RNA and protein levels, enhancement by the polyamine may be underestimated when eGFP is used as a transgene. To determine this, we measured the amount of BMP2 secreted into the culture supernatant after transduction of hBM-MSCs with Ad5BMP2, in the presence or absence of GeneJammer. Expression and secretion of BMP2 in hBM-MSCs were enhanced after combined GeneJammer–Ad5BMP2 transduction. Culture supernatants were collected 72 hr after initial transduction and BMP2 was quantified by enzyme-linked immunosorbent assay (ELISA). The results (Fig. 3A) show increased BMP2 protein in culture supernatant taken from hBM-MSCs transduced in the presence of the polyamine compared with hBM-MSC cultures from which GeneJammer was omitted during transduction. BMP2 was not detected in the culture supernatant from either cells alone or those transduced with the control virus Ad-empty, demonstrating that BMP2 expression in all cases specifically corresponded with viral transduction (Fig. 3A).

BMP2 protein in culture supernatant was further assayed for functional activity, using the W20-17 cell-based assay (Thies *et al*., 1992; Blum *et al*., 2001; Olmsted *et al*., 2001). In this assay, the murine bone marrow cell line W20-17 was exposed to culture supernatant from hBM-MSCs that had been transduced in the presence or absence of GeneJammer. This cell line responds to exogenously added BMP2 by increasing the expression of alkaline phosphatase. Culture supernatants from

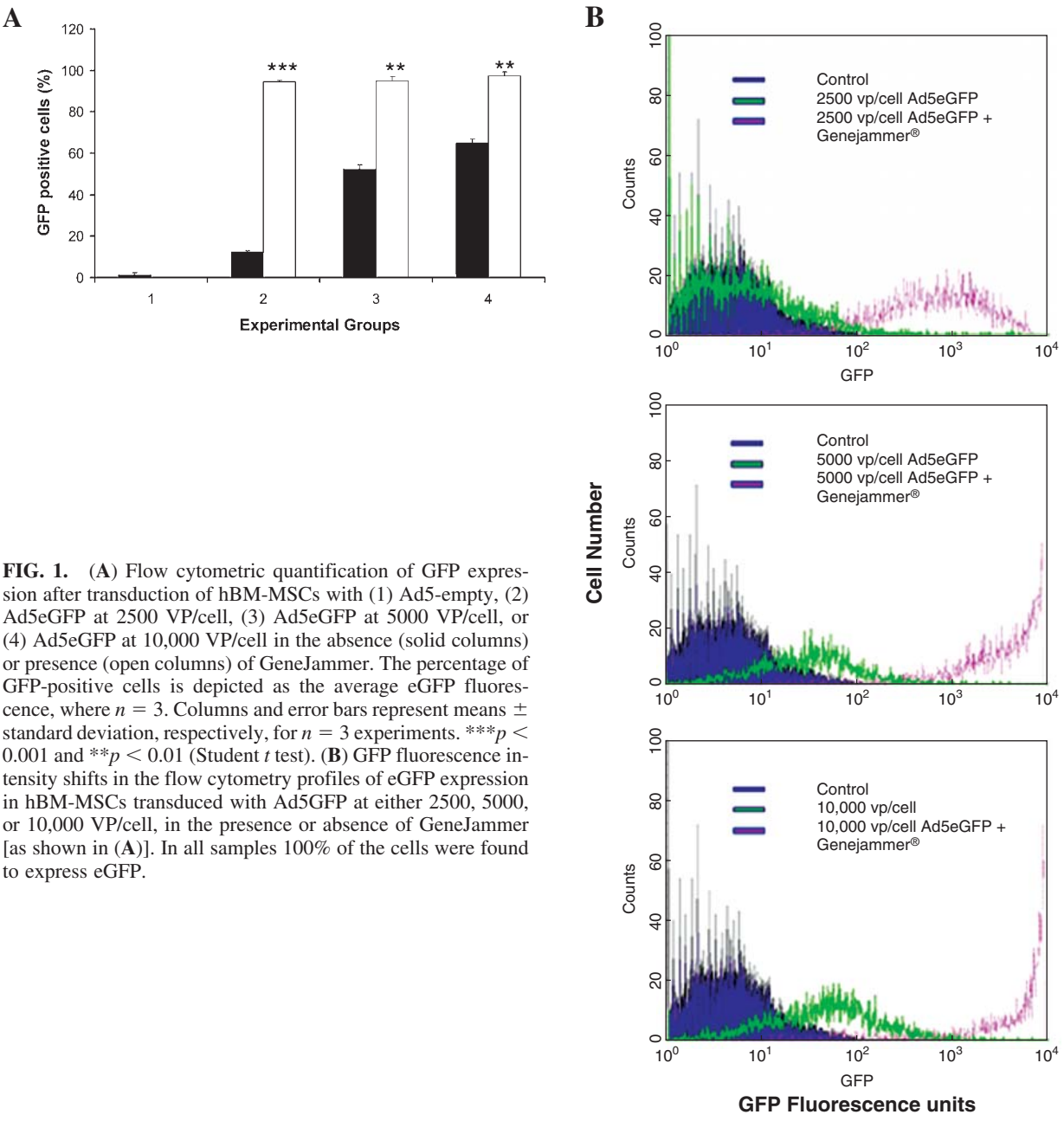

FIG. 1. (**A**) Flow cytometric quantification of GFP expression after transduction of hBM-MSCs with (1) Ad5-empty, (2) Ad5eGFP at 2500 VP/cell, (3) Ad5eGFP at 5000 VP/cell, or (4) Ad5eGFP at 10,000 VP/cell in the absence (solid columns) or presence (open columns) of GeneJammer. The percentage of GFP-positive cells is depicted as the average eGFP fluorescence, where $n = 3$. Columns and error bars represent means $\pm$ standard deviation, respectively, for $n = 3$ experiments. $^{***}p < 0.001$ and $^{**}p < 0.01$ (Student t test). (**B**) GFP fluorescence intensity shifts in the flow cytometry profiles of eGFP expression in hBM-MSCs transduced with Ad5GFP at either 2500, 5000, or 10,000 VP/cell, in the presence or absence of GeneJammer [as shown in (**A**)]. In all samples 100% of the cells were found to express eGFP.

hBM-MSCs and from cells transduced with the Ad-empty control virus were also included in the analysis to verify that the W20-17 response was BMP2 specific. As can be seen in Fig. 3B, we observed a 12-fold increase in relative chemiluminescence in the supernatant of W20-17 cells exposed to medium from hBM-MSCs transduced with Ad5BMP2 (2500 VP/cell) in the presence of GeneJammer as compared with those samples transduced in the absence of the compound. At virus concentrations of 5000 and 10,000 VP/cell, we observed a 4.5- and 1.8-fold increase, respectively, in the cells transduced in the presence of the polyamine (Fig. 3B). Alkaline phosphatase activity in samples exposed to the culture supernatant of cells transduced in the presence of GeneJammer were maximally activated, whereas exposure to culture supernatant of cells transduced in the absence of the polyamine appeared to increase with viral dose (Fig. 3B).

We have previously shown that transduction of hBM-MSCs with Ad5BMP2 induces little to no detectable bone formation whereas those cells transduced with Ad5F35BMP2 elicited significant bone formation in the same 2-week time period, suggesting that efficiency of cell transduction was critical to bone formation *in vivo* (Olmsted-Davis *et al*., 2002). To further confirm that GeneJammer can significantly increase Ad5BMP2 transduction efficiency and BMP2 production in hBM-MSCs without altering cell viability *in vivo*, we chose to compare these two cell populations in our heterotopic bone assay (Olmsted-Davis *et al*., 2002). Briefly, hBM-MSCs (5×10^6) transduced with either Ad5BMP2 or the control vector Ad5-empty in the

Table 1. Adenovirus Receptor Expression on Selected Cell Lines[a]

	Expression (%) of cell surface receptor		
Cell line	*CAR*	*Integrin α_v*	*CD46*
A549	93.28 ± 1.28	99.26 ± 0.53	99.27 ± 0.79
hBM-MSCs	0.34 ± 0.50[b]	87 ± 4.70[b]	31.78 ± 3.54
CHO	1.77 ± 0.27	2.04 ± 0.16	0.19 ± 0.05

Abbreviations: CAR, coxsackievirus–adenovirus receptor; CHO, Chinese hamster ovary; hBM-MSCs, human bone marrow mesenchymal stem cells.

[a]Numbers represent the mean percentage of cells expressing each receptor ± SD ($n = 3$).

[b]From Olmsted-David *et al.* (2002).

presence or absence of GeneJammer were injected into the hindlimb quadriceps muscle of NOD/SCID mice. After 2 weeks, the injected tissues were isolated and bone formation was determined by radiological analysis. As can be seen in Fig. 3C, mice that received cells transduced with Ad5BMP2 in the presence of GeneJammer had detectable mineralized bone, whereas those that received cells transduced in the absence of the polyamine had none. In no cases did we detect bone in mice receiving cells transduced with the Ad-empty control vector (data not shown). The data suggest that bone formation correlated with enhanced transduction efficiency and expression of BMP2.

We next determined the effect of GeneJammer on adenovirus transduction of the murine cell line MC3T3-E1. MC3T3-E1 is a C57BL/6-derived cell line that can be propagated *in vitro*, and used *in vivo* in C57BL/6 mice without eliciting a graft-versus-host response in the animals. However, they do not possess CAR or CD46 (data not shown), and therefore are not readily transduced with Ad5 or Ad5F35 vectors. To overcome this barrier the cells were transduced with Ad5BMP2 at various concentrations (2500, 5000, and 10,000 VP/cell) in the presence or absence of GeneJammer. Culture supernatant was removed approximately 72 hr after transduction and assayed for functional BMP2 activity, using W20-17 cells. Supernatant from cells transduced with Ad5BMP2 at 5000 VP/cell, in the presence of GeneJammer, yielded a significant elevation (approximately 2.5-fold) in alkaline phosphatase activity as compared with similar amounts of culture supernatant taken from parallel samples of cells transduced in the absence of GeneJammer (Fig. 4A). Further, the level of alkaline phosphatase induction was similar to that obtained with culture supernatant removed from hBM-MSCs after transduction with Ad5F35-BMP2 (Fig. 4A).

We have observed that we can make copious amounts of bone in immunodeficient NOD/SCID mice (Olmsted-Davis *et al*, 2002; Gugala *et al*, 2003) on injection of various human cell types transduced with AD5F35BMP2. However, in our current investigations we wished to inject transduced cells into immunocompetent mice. To aid in this process we transduced and injected murine cells (MC3T3-E1) rather than human cells. Approximately 5×10^6 MC3T3-E1 cells were transduced with either Ad5BMP2 (5000 VP/cell) or Ad5-empty (5000 VP/cell) in the presence or absence of GeneJammer and injected into the hindlimb quadriceps muscle of C57BL/6 mice ($n = 4$). Figure 4B shows the results of radiological analysis of the hindlimbs of only two of the mice, because all mice gave identical results, approximately 2 weeks after injection of the transduced cells. Mice that received cells transduced with Ad5BMP2 in the presence of GeneJammer displayed detectable mineralization whereas cells transduced in the absence of the polyamine showed little to no detectable bone. These findings demonstrate the ability of BMP2-transduced cells to elicit bone formation

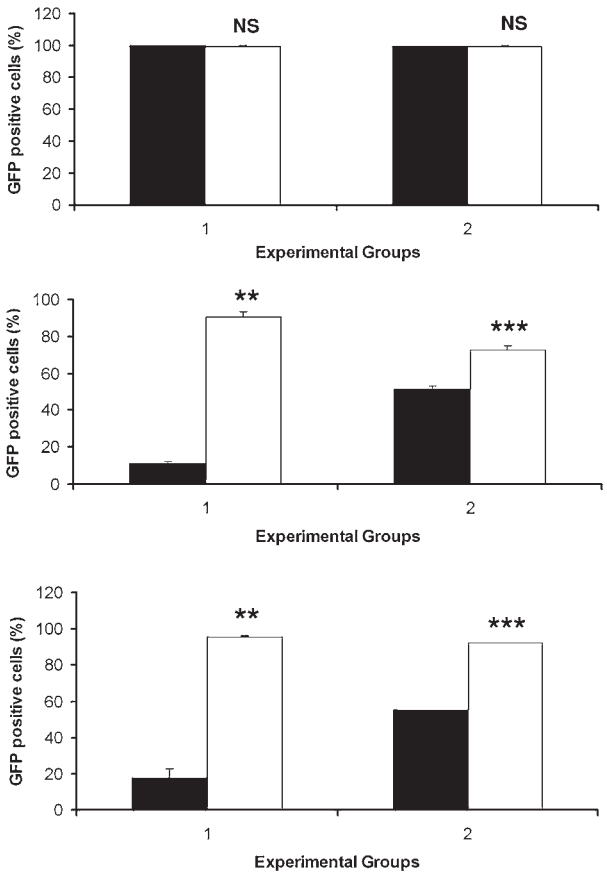

FIG. 2. Flow cytometry analysis of GFP expression of A549 cells (**A**), hBM-MSCs (**B**), and CHO cells (**C**) transduced with Ad5eGFP at 2500 VP/cell (group 1) or with Ad5F35eGFP at 2500 VP/cell (group 2) in the absence (solid columns) or presence (open columns) of GeneJammer. The percentage of GFP-positive cells is depicted as the average GFP fluorescence, where $n = 3$. Columns and error bars represent means ± standard deviation, respectively, for $n = 3$ experiments. $^{***}p < 0.001$ and $^{**}p < 0.01$ (Student t test).

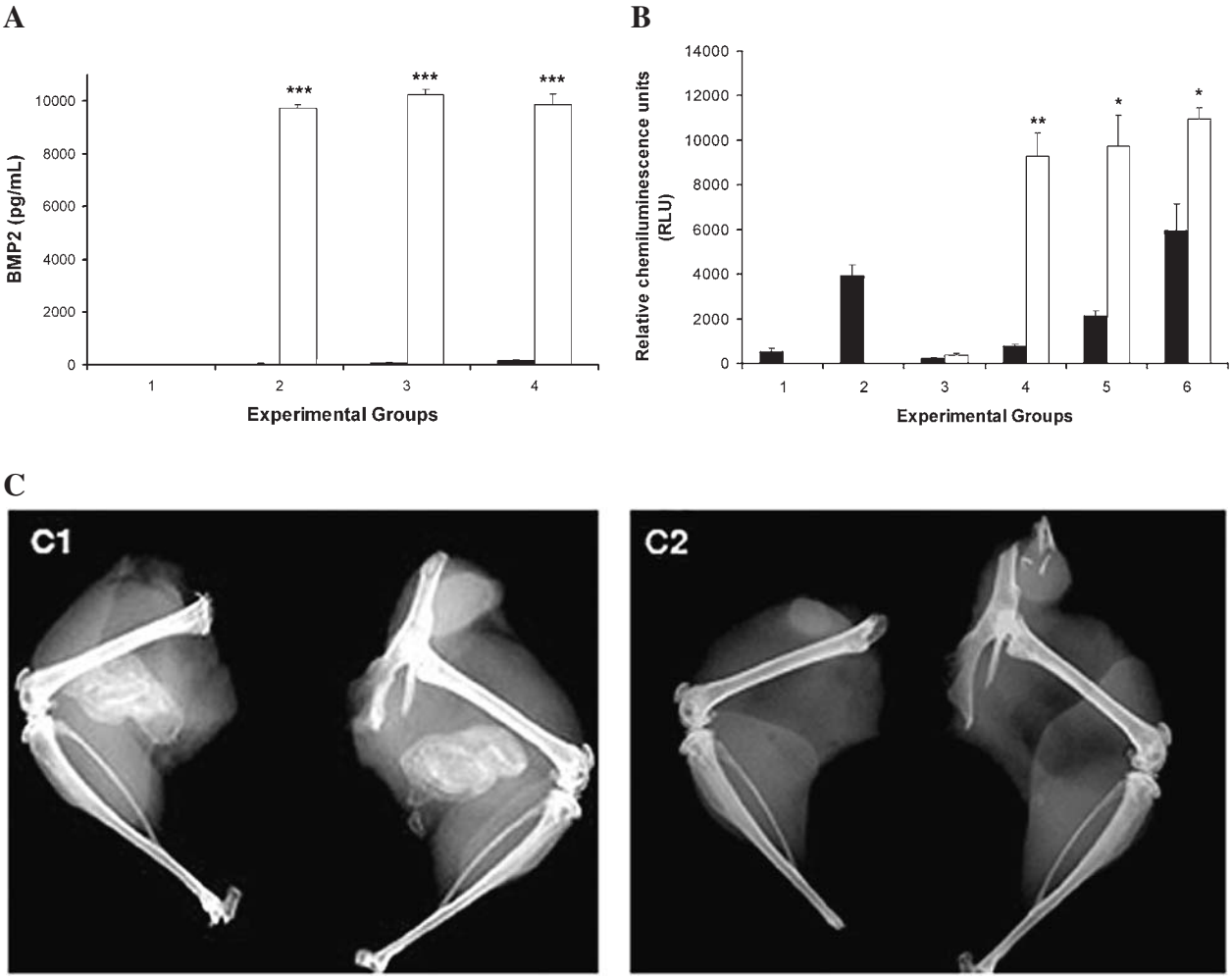

FIG. 3. Quantification of BMP2 protein (**A**), and activity *in vitro* (**B**) or *in vivo* (**C**). (**A**) BMP2 protein concentration was determined in culture supernatants, using the Quantikine BMP2 assay (R&D Systems). BMP2 activity was measured in culture supernatant taken from hBM-MSCs alone (group 1) and from hBM-MSCs transduced with Ad5BMP2 at 2500 VP/cell (group 2), 5000 VP/cell (group 3), and 10,000 VP/cell (group 4) in the absence (solid columns) or presence (open columns) of GeneJammer. The concentration of BMP2 was extrapolated from a standard curve based on known concentrations of recombinant BMP2. BMP2 concentrations in the supernatant are reported as picograms per milliliter, $n = 3$. $^{***}p < 0.001$ (Student t test). (**B**) BMP2 activity was measured in culture supernatant taken from hBM-MSCs alone (group 1), from hBM-MSCs transduced with control Ad5F35BMP2 (2500 VP/cell) (group 2), from hBM-MSCs transduced with Ad5-empty (5000 VP/cell) (group 3), or from hBM-MSCs transduced with Ad5BMP2 at 2500 VP/cell (group 4), 5000 VP/cell (group 5), or 10,000 VP/cell (group 6) by determining the increase in alkaline phosphatase activity in W20-17 cells 72 hr after exposure, in the absence (solid columns) or presence (open columns) of GeneJammer. Alkaline phosphatase activity is depicted as average relative chemiluminescence units (RLU), where $n = 3$. Columns and error bars represent means ± standard deviation, respectively, for $n = 3$ experiments. $^{**}p < 0.01$ and $^{*}p < 0.05$ (Student t test). (**C**) Radiologic analysis of heterotopic bone formation in NOD/SCID mice after intramuscular injection of hBM-MSCs transduced with Ad5BMP2 (5000 VP/cell) plus GeneJammer (panel C1) or Ad5BMP2 alone (panel C2).

even in mice with a normal immune system. Further, the results suggest once again that the level of Ad5BMP2 transduction of the cells is crucial to elicit bone formation.

DISCUSSION

Much emphasis in tissue engineering of bone has been focused on developing gene therapy approaches to induce bone formation. However, many critical obstacles still stand in the pathway of developing such a system for clinical use, such as efficient transduction of either delivery cells or target tissues, sequestration of the cell or vector system to the specific site, and avoidance of immune surveillance and clearing before obtaining the desired bone induction.

We and others have taken a virus-based gene therapy approach to deliver BMP2 ectopically for induction of new bone formation (Olmsted-Davis *et al*., 2002; Gugala *et al*., 2003; Tsuda *et al*., 2003; Zhu *et al*., 2004). Because BMP2 is a secreted protein, the transduced cells function as a delivery vehi-

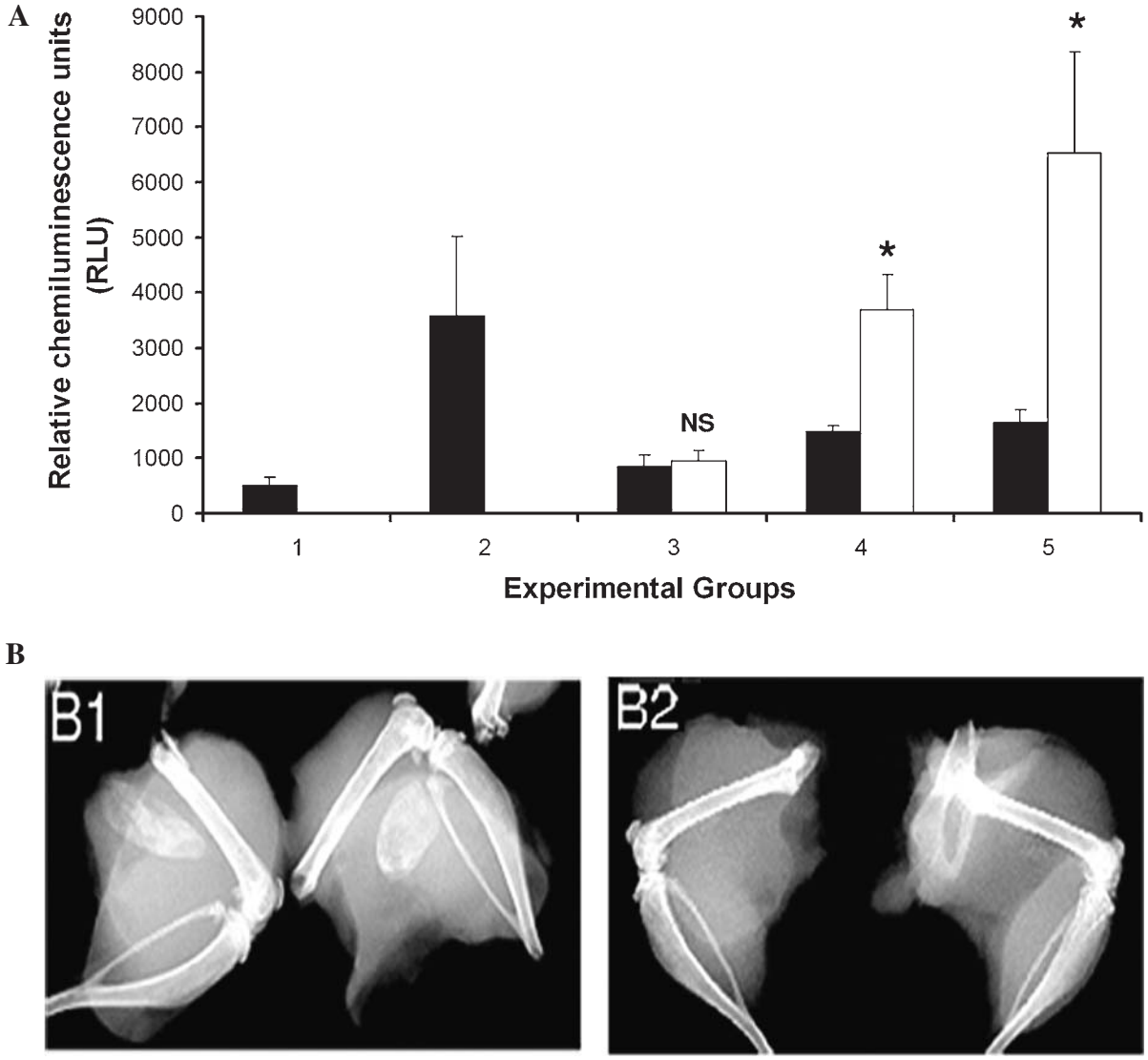

FIG. 4. Analysis of BMP2 activity both *in vitro* and *in vivo*. (**A**) BMP2 activity was analyzed in culture supernatant taken from MC3T3-E1 cells (group 1), from MC3T3-E1 cells transduced with Ad5F35BMP2 (2500 VP/cell) (group 2), and from MC3T3-E1 cells transduced with Ad5BMP2 at 2500 VP/cell (group 3), 5000 VP/cell (group 4), or 10,000 VP/cell (group 5), in the absence (solid columns) or presence (open columns) of GeneJammer. Alkaline phosphatase activity is depicted as average relative chemiluminescence (RLU), where $n = 3$. Columns and error bars represent means ± standard deviation, respectively, for $n = 3$ experiments. $^*p < 0.05$ (Student t test). (**B**) *In vivo* analysis of BMP2 activity was determined radiologically in C57BL/6 mice that received MC3T3-E1 cells transduced with Ad5BMP2 (5000 VP/cell) in the presence (panel B1) or absence (panel B2) of GeneJammer.

cle (Gugala *et al*., 2003), but they provide additional safety because no free virus is delivered to patients, which reduces both immune clearance and acute reaction to the virus itself. Ideally these cells would be readily obtainable from patients, such as from the peripheral blood, which would avoid additional immune response to foreign cells. However, we have previously demonstrated that bone formation was linked to the level of BMP2 secretion, with high levels of BMP2 inducing more bone formation than low levels. Hence transduction methodology must be versatile enough to provide high-level transduction of a variety of species required for preclinical development, as well as the cell types found in human blood.

Here we have presented a rapid, easy, inexpensive, and versatile method for achieving high-level adenovirus transduction of cells from various species, by inclusion of the DNA transfection reagent GeneJammer. Comparison of eGFP expression after transduction with Ad5eGFP or Ad5F35eGFP showed a significant increase in both the number of cells and the intensity of transgene expression in the presence of GeneJammer, independent of fiber type. Interestingly, in experiments in which 100% of the cells were transduced at all viral doses in the presence of GeneJammer, we observed an increase in GFP intensity corresponding with increasing amounts of adenovirus. As can be seen in Fig. 1B, at 2500 VP/cell, 100% of the cells are

transduced. However, the mean intensity of eGFP expression is approximately 10^3 eGFP fluorescence units, whereas at 5000 VP/cell the mean is greater than 10^4 eGFP fluorescence units. Further, at the highest virus concentration (10,000 VP/cell) approximately 60% of the cells express 10^4 eGFP fluorescence units, or more, as compared with the lower dose (5000 VP/cell), at which only 30% of the cells express this level of eGFP intensity. These results suggest that the mechanism by which GeneJammer assists adenovirus entry into cells is not limiting at the doses studied and that it can also increase the total number of adenovirus particles entering any individual cell, resulting in higher levels of expression per cell.

Because adenovirus fiber protein is involved in receptor binding and internalization, two likely models exist as to how the compound enhances cellular transduction. First, GeneJammer may bind the virus and allow cellular entry through either an alternative receptor or a non-receptor-mediated pathway. Alternatively, the compound may enhance adenovirus binding to its normal receptor, functioning as a coreceptor.

To determine which of the two tentative mechanisms was involved, we selected three different cell lines that had various levels of adenovirus receptors and transduced them in the presence or absence of GeneJammer. As expected, Ad5eGFP was able to infect 100% of the CAR-positive cell line A549, but was less efficient at transducing hBM-MSCs (which possess only the α_v receptor), with 10% of the cells positive for eGFP expression. Further, in CHO cells, which are negative for both adenovirus-specific receptors, we observed no cells expressing eGFP. However, when GeneJammer was included in the transduction reaction, approximately 100% of all three cell types expressed the transgene. Significant enhancement of expression was achieved in the cell lines that lacked one or both adenovirus type 5-specific receptors, whereas in the receptor-positive cell line the transduction efficiency remained the same, presumably because it was maximal under both circumstances. Interestingly, the results suggest that GeneJammer is not enhancing viral entry through its normal receptor/coreceptor-mediated route but, rather, is using a novel mechanism, because 100% of the cells lacking adenovirus type 5-specific receptors were transduced. This finding is consistent with other studies in which uptake of adenovirus via hexon protein binding to dipalmitoyl phosphatidylcholine resulted in cellular internalization via an alternative mechanism that does not use receptor binding (Balakireva *et al*., 2003). However, the authors suggested that this is a mechanism used by adenovirus for entry into the lung, because alveolar epithelial type II cells synthesize the major component of pulmonary surfactant, disaturated phosphatidylcholine, on adenovirus infection. Further, they suggested that although the cells do possess adenovirus-specific receptors, it is the production of this compound that leads to the high transduction efficiency found with these cell types, including A549 cells (Balakireva *et al*., 2003). Further studies are required to determine whether GeneJammer functions through similar mechanisms.

GeneJammer significantly enhanced expression of eGFP when Ad5F35eGFP was used in the transductions. Again, this suggests that the mechanism by which GeneJammer enhances virus entry into the cell is not specific to fiber type 5. As expected, 100% of A549 cells, which possess the adenovirus type 35-specific receptor CD46, were transduced in the presence or absence of the compound. Interestingly, we observed approximately the same transduction efficiency with both hBM-MSCs and CHO cells when using Ad5F35eGFP, even though these cell lines differ significantly in the level of CD46. One possible explanation is that the anti-human CD46 antibody used in these experiments may not detect hamster CD46. The lower level of induction by GeneJammer is in part due to the higher transduction efficiency of the Ad5F35eGFP vector in its absence, with approximately 55–60% of the cells transduced. Ad5eGFP transduction of CHO cells, however, is low in the absence of GeneJammer, and it has been shown previously (Bergelson *et al*., 1997; Davison *et al*., 1999) that CHO cells lack CAR, because CHO cells can be transduced by Ad5 vectors when these cells are transfected with a vector that elicits the expression of CAR.

We next measured the level of BMP2 production in cells transduced with Ad5BMP2 to determine whether GeneJammer could enhance the expression of this protein. As seen in Fig. 3A–C, there was a significant enhancement in BMP2 production and activity *in vitro* and *in vivo*. Interestingly, in the assay for functional BMP2, medium collected from hBM-MSCs transduced with Ad5F35BMP2 stimulated alkaline phosphatase to a level of approximately 5000 chemiluminescence units, whereas the same cells transduced with Ad5BMP2 in the presence of GeneJammer elevated alkaline phosphatase to a level of 9000 chemiluminescence units, demonstrating that this universal system could actually lead to increased BMP2 activity as compared with a parallel system using the chimeric Ad5F35BMP2 (Olmsted-Davis *et al*., 2002). As expected, this high-level expression of BMP2 after transduction of hBM-MSCs with Ad5BMP2 in the presence of GeneJammer led to significant *in vivo* bone formation after implantation, whereas the cells transduced in parallel in the absence of GeneJammer did not induce detectable mineralization or osteoid by histology. Again, this verifies our previous finding (Gugala *et al*., 2003) that the level of BMP2 expression dictates the formation of bone and is linked to the efficiency of transduction of the cells. Hence developing a system that would routinely provide high-level transduction of cells with adenovirus, independent of vector type, is an essential component in developing a gene therapy system to induce bone formation. Further, the data also demonstrate that there are no apparent deleterious effects on these processes or the BMP2 protein itself as a result of the potential presence of residual compound in the medium or to the animals after injection of these transduced cells, suggesting that this is safe for conducting preclinical trials.

We next determined the effects of this compound on the efficiency of Ad5BMP2 transduction of a murine cell line, MC3T3-E1, by measuring BMP2 activity in the culture supernatant, using the W20-17 assay. Previous attempts to achieve high-level transduction of this cell line have failed, partly because of the lack of adenovirus-specific receptors for either the Ad5 or Ad5F35 vector (Mallam *et al*., 2004). However, use of this or other syngeneic murine cells is critical in preclinical studies using Ad5BMP2-transduced cells, because it is derived from the C57BL/6 strain and does not elicit a graft-versus-host reaction in recipient C57BL/6 mice (data not shown). The results demonstrate that GeneJammer provides a similar enhancement of expression in these murine cells as was observed in hBM-MSCs. Interestingly, at 2500 VP/cell neither transduc-

tion methodology led to detectable levels of BMP2 protein in the culture supernatant, presumably because of potential low-level transduction of the MC3T3-E1 cells. However, at the higher doses of Ad5BMP2 we did observe significant increases in alkaline phosphatase activity correlating with BMP2 activity in the culture supernatant, presumably because of increased transduction of the MC3T3-E1 cells in the presence of GeneJammer. As can be seen in Fig. 4A, culture supernatant taken from MC3T3-E1 cells transduced with Ad5BMP2 (5000 or 10,000 VP/cell) in the presence of GeneJammer led to at least the same or higher levels of alkaline phosphatase and/or BMP2 activity than was achieved with culture supernatant collected from Ad5F35BMP2 (2500 VP/cell)-transduced hBM-MSCs (Fig. 3B), which consistently produced the level of BMP2 necessary to induce bone formation *in vivo*. These results suggest that the cells are transduced to express BMP2 at a level capable of eliciting bone formation in immune-incompetent mice. The lowest level of BMP2 production we have used to successfully form bone *in vivo* in immune-incompetent and immune-competent mice are 9 and 18 ng of BMP2 per 10^6 cells per day, respectively. As can be seen in Fig. 4B, when MC3T3-E1 cells transduced with Ad5BMP2 (5000 VP/cell) in the presence of GeneJammer were implanted *in vivo* into C57BL/6 mice, we observed significant bone formation, suggesting that this level of BMP2 is also sufficient to induce bone formation in immunocompetent mice. However, when samples transduced in parallel but in the absence of GeneJammer were implanted we did not observe any bone formation, again presumably because of the low level of BMP2 expression from these cells. Further, these results suggest that high-level BMP2 production is essential regardless of the immune profile of the mice. Interestingly, this is contradictory to a previous report that suggests that low-level virus transduction of cells with potent osteoinductive agents may prevent significant immune response, allowing longer transgene expression and greater bone induction (Kim *et al*., 2003). In our system the BMP2-transduced cells are eliminated within 4 days yet we still observed robust bone formation, suggesting that avoidance of the immune system is not essential.

We conclude that the use of GeneJammer as described in this study can significantly enhance adenovirus entry into cells even in those lacking adenovirus-specific receptors. Thus this technique provides investigators the ability to transduce a wide range of cells by a rapid, inexpensive method, offering substantial versatility that was previously lacking. Such advances are critical in the translation of cell-based gene therapy systems from small animal models into preclinical and clinical testing.

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